REMARKS

This paper is a Response to the Office Action mailed July 31, 2007. Claims 111 to 134 are under consideration.

Regarding the Claim Amendments

The amendments to the claims are supported throughout the specification. In particular, the amendment to recite "antigen binding" fragment is supported, for example, at page 12, lines 25-28. The amendment to claim 127 to recite inhibits proliferation of "adenocarcinoma cells of the pancreas in vitro" is supported, for example at page 56, lines 8-29, and page 57, line 28, to page 58, line 15. Thus, as the claim amendments are supported by the specification, no new matter has been added and entry thereof is respectfully requested.

Regarding the Priority Applications

Submitted herewith are certified English translations of the three German priority applications, namely DE 102 29 906.4, DE 102 29 907.2 and DE 102 30 516.1. Support for the claims, as amended, can be found throughout each of the priority applications. Accordingly, in view of the submission of the certified English translations, the subject application has a July 4, 2002, priority date.

Regarding the Information Disclosure Statement

Applicants note that certain references submitted in an IDS and listed on PAT-1449 were not considered by the Examiner since they were not in the English language. Submitted herewith is an Information Disclosure Statement with copies of publications in English that appear to be equivalent or are related by a claim of priority to the foreign language publications that were not considered. Reference DE 4107154A1 (reference KR, PAT-1449) was listed as a priority application for U.S. Patent No. 5,610,280. Applicants therefore respectfully request consideration of US Patent No. 5,610,280 on the attached PAT-1449. Reference DE 10230516A1 (reference LR, PAT-1449) was published January 1, 2004, and is therefore not prior art. Reference DE 69212671T2 (reference MR, PAT-1449) is a German translation that lists European patent application 92810056.9. A copy of this European patent application, namely Publication 0502812A1, is submitted herewith and Applicants

respectfully request consideration thereof. Reference DE 69229110T2 (reference NR, PAT-1449) is a German translation that lists PCT publication WO 92/16624. A copy of this PCT publication is submitted herewith and Applicants respectfully request consideration thereof. Reference DE 69527975T2 (reference OR, PAT-1449) is a German translation that lists PCT publication WO 96/16990. A copy of this PCT publication is submitted herewith and Applicants respectfully request consideration thereof. Reference EP 1141019 B1 (reference RR, PAT-1449) is an issued EP patent in German based upon PCT WO 00/37489. PCT WO 00/37489A2 and A3 were both submitted in the IDS (references UR and TR, respectively, PAT-1449), and were considered by the Examiner. Consequently, EP 1141019 B1 is cumulative with respect to the references PCT WO 00/37489A2 and A3 that have been considered. An English translation of the Timmerman *et al* reference (reference AAAAR, PAT-1449) is submitted in the Information Disclosure Statement and Applicants respectfully request consideration thereof.

The above-identified English publications are listed on the attached Form PAT-1449. Applicants respectfully request consideration of the accompanying English publications and that the Examiner return an initialed copy of Form PAT-1449 to the undersigned indicating that the publications have been considered.

I. REJECTION UNDER 35 U.S.C. §112, SECOND PARAGRAPH

Claims 111 to 130 stand rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite. Allegedly, the claims are indefinite because of the recitation of 55 or 110 kDa molecular weight, and whether denaturing or non-denaturing conditions were used for the molecular weight determination.

Claims 111 to 130 are clear and definite under 35 U.S.C. §112, second paragraph. In terms of why the antibody binds to polypeptides having different molecular weights, there are a variety of possibilities. In this regard, without being bound by any particular possibility the molecular weight of intact protein could be 110 kDa which, if degraded during preparation or size fractionation by electrophoresis, forms a 55 kDa fragment. Thus, the antibody could bind to both 55 and 110 kDa molecular weight forms if the epitope is present on the intact 110 kDa protein and the 55 kDa degradation product. Another possible explanation is that the 110 kDa form represents an unprocessed protein containing the epitope, whereas the 55 kDa form represents a proteolytically processed (cleaved) version of the 110 kDa protein; again the antibody can bind to 55 and 110 kDa molecular weight forms since the epitope is

present on both forms. There are other possible explanations, all of which would be known to the skilled artisan. Given that there are several plausible explanations why the antibody binds to different molecular weight proteins, which explanations would be known to the skilled artisan, the claims are not unclear or indefinite due to the recitation of multiple molecular weights.

In terms of whether denaturing or non-denaturing conditions were used for the molecular weight determination, as is known in the art sodium dodecyl sulfate is a detergent. Thus, in view of the fact that a detergent was used for size fractionation, the skilled artisan would know that the molecular weight determination was under denaturing conditions. Given that the skilled artisan would know that denaturing conditions were used for molecular weight determination, the claims are clear and definite.

In view of the foregoing, claims 111 to 130 are clear and definite. Consequently, the rejection under 35 U.S.C. §112, second paragraph is improper and must be withdrawn.

II. REJECTIONS UNDER 35 U.S.C. §112, FIRST PARAGRAPH

The objection to the specification and rejection of claim 134 under 35 U.S.C. §112, first paragraph, as allegedly lacking an adequate written description and enablement is respectfully traversed. The grounds for rejection are set forth in the Office Action, pages 4-5.

Access to the deposited PM-2 cell line will be made in accordance with the provisions of 37 C.F.R. §1.808. Accordingly, as the PM-2 deposit will be made available to the public according to 37 C.F.R. §1.808 upon issuance of the patent, the grounds for rejection are moot and Applicants respectfully request that withdrawal of the rejection.

The rejection of claims 111 to 130 under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement is respectfully traversed. The grounds for rejection are set forth in the Office Action, pages 5-22.

The proper standard for enablement under 35 U.S.C. §112, is whether one skilled in the art could make and use the invention without undue experimentation. In this regard, "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *In re Wands* 858 F.2d 731, 737 (Fed. Cir. 1988)

Here, in view of the guidance in the specification and knowledge and skill in the art concerning antibody structure and function at the time of the invention, and that antibody variants having the requisite activity could be produced and identified using routine methods disclosed in the specification or that were known in the art at the time of the invention, one skilled in the art could make antibodies and antigen binding fragments that specifically bind a polypeptide having an approximate molecular weight of 55 or 115 kDa using sodium dodecyl sulfate polyacrylamide gel electrophoresis expressed by ASPC-1 (ATCC Accession No. CRL-1682) and BXPC-3 (ATCC Accession No. CRL-1687) which comprises a sequence at least 80% identical to the sequence of SEQ ID NO:5 or comprises a sequence at least 80% identical to the sequence of SEQ ID NO:7 without undue experimentation.

First, the claims have been amended to recite that the fragment is an antigen binding fragment. Thus, the ground for rejection due to an Fc or other fragment that may not bind to antigen is moot. Furthermore, claim 127 has also been amended to recite inhibition of adenocarcinoma cells of the pancreas in vitro. Thus, the ground for rejection due to inhibiting proliferation of cell types other than adenocarcinoma cells of the pancreas is moot.

Second, the Examiner has acknowledged that the level of knowledge and skill with respect to antibody structure and function at the time of the invention was high. For example, as discussed at length in the Office Action the role of antibody heavy and light chain variable regions, particularly CDRs and FRs, in antigen binding were well understood by the skilled artisan at the time of the invention (see, for example, pages 9-11 of the Office Action). The specification also discloses the role of antibody heavy and light chain variable regions, CDR and FR regions in antigen binding (page 22, line 6, to page 23, line 2). Consequently, in view of the high level of knowledge and skill in the art with respect to antibody structure and function at the time of the invention clearly the skilled artisan would be apprised of antibody regions that participate in antigen binding.

In addition to the high level of knowledge and skill in the art with respect to antibody structure and function, as acknowledged by the Examiner the specification discloses the locations of the CDRs in SEQ ID NOs:5 and 7 (page 7 of the Office Action). In particular, the specification discloses the CDRs in SEQ ID NOs:5 and 7 in Figures 14 and 15 (see, also, pages 5, lines 6-7 and 24-25). Furthermore, in view of the fact that the specification discloses the location of the CDRs in SEQ ID NOs:5 and 7 and that SEQ ID NOs:5 and 7 are human sequences, the skilled artisan would know the location of the FRs in SEQ ID NOs:5 and 7.

Consequently, in view of the guidance in the specification the skilled artisan would know the location of CDRs and FRs of SEQ ID NOs:5 and 7.

Because the knowledge and skill in the art at the time of the invention was high in terms of antibody structure and function and the location of sequences in SEQ ID NOs:5 and 7 that contribute to antigen binding would be known, the skilled artisan would also know residues in SEQ ID NOs:5 and 7 that would be amenable to substitution and therefore, be able to predict with reasonable certainty variants of SEQ ID NOs:5 and 7 that would have at least partial binding activity. As a non-limiting example illustrating this point, the skilled artisan would know that an amino acid substitution, such as a conservative substitution, for example, outside of a CDR or FR region of in SEQ ID NOs:5 and 7 would likely not destroy antigen binding activity. Thus, the skilled artisan could make a conservative substitution of either SEQ ID NOs:5 or 7 outside of a CDR or FR with a reasonable certainty that the substituted sequence would retain at least partial antigen binding activity. Given the large number of amino residues outside of CDR and FR regions, and the number of amino residues outside of antibody variable regions, clearly many variants of SEQ ID NOs:5 and 7 could be readily produced that have at least partial antigen binding activity. As an additional non-limiting example illustrating this point, the skilled artisan would know that given the role of CDRs in antibody binding a large number of non-conservative amino acid substitutions in the CDRs in SEQ ID NOs:5 and 7 would likely reduce or eliminate antigen binding. Thus, the skilled artisan would know not to delete or introduce a large number of non-conservative substitutions into the CDRs in SEQ ID NOs:5 and 7. Consequently, in view of the guidance in the specification and the high level of knowledge and skill in the art regarding antibody structure and function, the skilled artisan would know of general regions and particular residues that would be more or less amenable to substitution and could therefore predict SEQ ID NOs:5 and 7 variants likely to have at least partial antigen binding activity without actually having to produce such variants and fragments.

In addition to knowing regions and residues of antibodies that would be more or less amenable to substitution or deletion, the level of knowledge and skill in the art regarding producing antibodies and antigen binding fragments thereof was also high. For example, methods of producing antibodies and variants without undue experimentation are disclosed in the specification (page 24, line 5, to page 28, line 24). Furthermore, methods of producing antibody fragments (*e.g.*, Fv, Fab, Fab' and $F(ab')_2$) were known in the art and were routine at the time of the invention. Methods of identifying antibody variants and fragments that

bind antigen without undue experimentation were also known in the art and are taught by the specification. In particular, routine methods for measuring antibody binding to antigen or cell lines, as well as methods for measuring cell proliferation and apoptosis are disclosed in the specification (page 45, line 24 to page 47, line 10; page 47, line 27, to page 49, line 14; page 56, lines 1-27; and page 57, line 19, to page 58, line 11). Thus, in view of the guidance in the specification and the high level of knowledge and skill in the art at the time of the invention regarding producing antibodies and antigen binding fragments, one skilled in the art could make antibodies and antigen binding fragments that specifically bind a polypeptide having an approximate molecular weight of 55 or 115 kDa using sodium dodecyl sulfate polyacrylamide gel electrophoresis expressed by ASPC-1 (ATCC Accession No. CRL-1682) and BXPC-3 (ATCC Accession No. CRL-1687) which comprises a sequence at least 80% identical to the sequence of SEQ ID NO:5 or comprises a sequence at least 80% identical to the sequence of SEQ ID NO:7 without undue experimentation.

Moreover, Applicants respectfully point out that if the skilled artisan wished to produce variants of SEQ ID NOs:5 or 7, producing recombinant proteins was routine in the art at the time of the invention, and the specification discloses routine assays for identifying antibodies that bind to the recited cell types, as discussed above. Analogous to *In re Wands,* 858 F.2d 731 (Fed. Cir. 1988), where the court held that screening hybridomas to determine those that produced monoclonal antibodies having a particular binding characteristic did not require undue experimentation, undue experimentation would not be required to identify antibody variants and fragments that bind to the recited cell types, given that 1) producing antibody variants and fragments was routine; and 2) cell binding and proliferation assays were routine at the time of the invention. Consequently, there is no need for the skilled artisan to "predict" in advance variants or fragments that bind to the recited antigen in order to make variants and antigen binding fragments. In view of the foregoing, the skilled artisan could produce antibody variants and antigen binding fragments without knowing *a priori* the effect of particular substitutions or deletions on activity.

Finally, the number of antibody variants and antigen binding fragments encompassed by the claims are limited as they are required to bind to antigen and therefore do not include inoperative embodiments. The claimed antibodies and fragments are further limited in number because of the high degree of sequence identity, namely at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7. Thus, the number of antibody variants and fragments encompassed by the claims will necessarily be limited based upon the functional

and structural requirements of antibodies, that the antibodies and fragments will have at least partial antigen binding activity, and that the antibodies and fragments will have a sequence at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7.

In view of the foregoing, the skilled artisan could make antibody variants and antigen binding fragments at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7 having the recited antigen binding activity without undue experimentation. Consequently, claims 111 to 130 are adequately enabled under 35 U.S.C. §112, first paragraph, and Applicants respectfully request that the rejection be withdrawn.

The rejection of claims 111 to 133 under 35 U.S.C. §112, first paragraph as allegedly lacking an adequate written description is respectfully traversed. Allegedly the claimed subject matter is not described in the specification to one skilled in the art that the inventor at the time the application was filed had possession of the claimed invention.

Claims 111 to 133 as originally filed are adequately described. Nevertheless, solely in order to further prosecution of the application and without acquiescing to the propriety of the rejection, the claims have been amended as set forth above. The rejection will therefore be addressed with respect to the amended and new claims.

A proper analysis for written description under 35 U.S.C. §112, first paragraph is whether one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563 (Fed. Cir. 1991); see, also, *Ralston Purina Co. v. Far-Mar-Co, Inc.*, 772 F.2d 1570, 1575 (Fed. Cir. 1985). To satisfy the written description requirement, "Applicants are not required to disclose every species encompassed by their claims, even in an unpredictable art." *In re Angstadt*, 537 F.2d 498, 502-503 (CCPA 1976), *Utter v. Hiraga*, 845 F.2d 993, 998-99 (Fed. Cir. 1988). Thus, a description of every antibody or antigen binding fragment is not required. Furthermore, the Federal Circuit recently held "that (1) examples are not necessary to support adequacy of a written description (2) the written description standard may be met (as it is here) even where actual reduction to practice of an invention is absent; and (3) there is no *per se* rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure." *Falkner v. Inglis*, 448 F.3d 1357 (Fed. Cir. 2006). Thus, in view of the standard set by the court, a genus can be adequately described under 35 U.S.C. §112, first paragraph without specific examples, an actual reduction to practice, or a complete structure of antibodies and functional fragments.

Here, in view of the guidance in the specification, which discloses antibody variable heavy and light chain sequences (e.g., SEQ ID NOs:5 and 7), and the high level of knowledge and skill in the art regarding structure and function of antibodies and antigen binding fragments the skilled artisan would be apprised of an adequate number of antibodies and antigen binding fragments within the genus of claims 111 to 133. Consequently, claims 111 to 133 are adequately described.

As discussed above, the specification teaches antibody heavy and light chain variable sequences (e.g., SEQ ID NOs:5 and 7). The specification also teaches the position of the three CDRs in each heavy and light chain variable region sequence, and therefore the position of the flanking regions (FR). In view of the foregoing guidance in the specification, one skilled in the art would know the location of the amino acid sequences that contribute to antigen binding.

As also discussed above, the level of knowledge and skill in the art with respect to antibody structure and function was high at the time of the invention. Evidence of such knowledge regarding antibody structure and function, such as native antibodies having two heavy and light chain sequence, the presence and contribution of three CDRs to binding, and the role of FRs is acknowledged in the Office Action and is taught by the specification. Thus, in view of the high degree of knowledge and skill in the art concerning antibody structure and function at the time of the invention, when combined with the guidance of the specification of the heavy and light chain variable sequences, SEQ ID NOs:5 and 7, the location of the CDRs and FRs that contribute to antigen binding, the molecular weights of the antigen and the cells types expressing the antigen, and the high degree of sequence identity to SEQ ID NOs:5 or 7, the skilled artisan would know variants of SEQ ID NOs:5 and 7 that would retain least partial antigen binding activity. As an illustration, the skilled artisan would know that a conservative substitution outside of a CDR or FR of either SEQ ID NOs:5 or 7 would retain at least partial antigen binding activity. Given the number of amino residues outside of the CDR and FR regions, and the large number of amino residues outside of antibody variable regions, clearly the skilled artisan could readily envision a number of antibody variants and antigen binding fragments within the scope of the claims that have at least partial antigen binding activity of SEQ ID NOs:5 and 7. Consequently, the skilled artisan would be apprised of a number of antibodies and antigen binding fragments within the scope of the claims.

In terms of a description of the claimed antibodies and antigen binding fragments to distinguish them from other materials, as discussed above the antibodies and antigen binding fragments are described 1) structurally- they have a high percentage of identity (at least 80%) to heavy or light chain variable sequences, SEQ ID NOs:5 and 7; and 2) functionally- they bind to a polypeptide having an approximate molecular weight of 55 or 110 kDa using SDS-PAGE, wherein the polypeptide is expressed by ASPC-1 and BXPC-3 cells. Thus, as the claimed antibodies and antigen binding fragments are described structurally- they have a heavy or light chain sequence with high degree of sequence identity to SEQ ID NOs:5 and 7, and functionally- they bind to an antigen specified by molecular weight and expressed on particular cells, the antibodies and antigen binding fragments are adequately distinguished from other materials.

In terms of the concern regarding a description of the antigen to which the antibodies bind, as discussed above the antigen is defined in terms of molecular weight. As also discussed above, the antigen to which the claimed antibodies bind is expressed by the specified cell types. Finally, the antigen is defined based upon its binding to antibody an antibody comprising SEQ ID NOs:5 and 7. Thus, the antigen can be considered described in view of the specified molecular weight, expression on the two specified cell types and the antibody to which the antigen binds. Furthermore, as discussed above the written description requirement may be satisfied without examples or an actual reduction to practice. In view of the fact that 35 U.S.C. §112, first paragraph does not require examples or an actual reduction to practice, clearly the written description requirement can be satisfied without actually isolating or sequencing the antigen to which the claimed antibodies and fragments bind.

Moreover, because the written description requirement under 35 U.S.C. §112, first paragraph may be satisfied without examples or an actual reduction to practice, the written description requirement can be satisfied if the skilled artisan knows of a number of antibody and antigen binding fragment of species within the claimed genus. Here, in view of the high level of knowledge and skill in the art with respect to antibody structure and function and the guidance in the specification as to the locations of CDRs and FRs in SEQ ID NOs:5 and 7 that participate in antigen binding, clearly the skilled artisan would readily envision a number of antibody and antigen binding fragment species within the claimed genus. Again, as discussed above, there are many amino residues outside of the CDR and/or FR regions, such that the skilled artisan could readily envision a number of antibody variants and antigen

binding fragments within the scope of the claims that have at least partial antigen binding activity.

In sum, in view of the guidance in the specification and the high level of knowledge and skill in the art at the time of the invention, the skilled artisan would be apprised of a representative number of antibodies and antigen binding fragments within claims 111 to 133. As such, an adequate written description of antibodies and antigen binding fragments is provided, and Applicants respectfully request that the rejection under 35 U.S.C. §112, first paragraph as allegedly lacking an adequate written description be withdrawn.

III. REJECTIONS UNDER 35 U.S.C. §102

The rejection of claims 111 to 123 and 126 to 134 under 35 U.S.C. §102(a) as allegedly anticipated by Brandlein *et al.* (Human Antibodies 11:107 (2002)) is respectfully traversed. Allegedly, Brandlein *et al.* describe each and every element claimed, as set forth on pages 34-40 of the Office Action.

Brandlein *et al.* (Human Antibodies 11:107 (2002)) was not published prior to July 4 or 6, 2002, the filing dates of the German priority applications. In support of Applicant's position, submitted herewith as Exhibit A is a copy of an email received from Ms. Susan Hendriks, marketing Coordinator at IOS Press, the publisher of the journal Human Antibodies. In the email, Ms. Susan Hendriks states that "Volume 11, number 4 of Human An[ti]bodies was published on April 18th 2003." Consequently, Brandlein *et al.* (Human Antibodies 11:107 (2002)) is not prior art against claims 111 to 123 and 126 to 134, and Applicants respectfully request that the rejection under 35 U.S.C. §102(a) be withdrawn.

The rejection of claims 111 to 123 and 126 to 134 under 35 U.S.C. §102(b) as allegedly anticipated by Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) as evidenced by Brandlein *et al.* (Human Antibodies 11:107 (2002)) is respectfully traversed. Allegedly, Brandlein *et al.* describe each and every element claimed, as set forth on pages 40-44 of the Office Action.

Anticipation requires the disclosure in a single prior art reference of each element of the claim under consideration *In re Spada*, 911 F.2d 705 (Fed. Cir. 1990), *In re Bond*, 910 F.2d 831 (Fed. Cir. 1990). Furthermore, a reference cited under section 102 must contain an enabling disclosure. *citations omitted*, see, M.P.E.P. §2121.

As a first issue, Applicants respectfully point out that a reference cited under 35 U.S.C. §102 must have an enabling disclosure. Thus, for this rejection to be proper, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) must enable claims 111 to 123 and 126 to 134. However, these claims have also been rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. Consequently, the rejections under 35 U.S.C. §102(b) and 35 U.S.C. §112, first paragraph are contradictory and cannot be maintained simultaneously. Applicants therefore respectfully request that the Patent Office withdraw either the rejection under 35 U.S.C. §102(b) or the rejection under 35 U.S.C. §112, first paragraph.

As a second issue, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) fail to teach or suggest antibodies or antigen binding fragments with a sequence at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7. In this regard, there is no sequence information for any antibody, let alone antibodies and antigen binding fragments at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7. Consequently, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) fails to teach or suggest the claimed antibodies.

Furthermore, as discussed above reference cited under 35 USC 102 must have an enabling disclosure and there is no antibody sequence described nor any method to obtain any antibody described in Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)). Absent antibody sequence or a method to obtain the antibody one skilled in the art could not produce the antibody. Consequently, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) fail to enable claims 111 to 123 and 126 to 134.

In sum, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) fail to teach or suggest and fail to enable claims 111 to 123 and 126 to 134. As such, the rejection under 35 U.S.C. §102(b) is improper and Applicants respectfully request that the rejection be withdrawn.

IV. REJECTION UNDER 35 U.S.C. §103(a)

The rejection of claims 124 and 125 under 35 U.S.C. §103(a) as allegedly obvious over Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) in view of Taylor *et al.* (US Patent 5,001,225) is respectfully traversed. Allegedly, Brandlein *et al.* in combination with the secondary reference teach or suggest each and every element claimed, as set forth on pages 45-47 of the Office Action.

As discussed above, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) fail to describe any antibody sequences, let alone antibodies and antigen binding fragments at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7. Consequently, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) fail to teach or suggest each and every element of claims 124 and 125.

Furthermore, without sequence information or a method to produce the antibody, one skilled in the art would not have a had a reasonable expectation of success at the time of the invention of producing an antibody or an antigen binding fragment at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7. Consequently, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) fail to provide a reasonable expectation of success in producing an antibody or an antigen binding fragment at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7 of claims 124 and 125.

The secondary reference of Taylor *et al.* (US Patent 5,001,225) fails to provide that which is missing from Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)). In this regard, there is no sequence described in Taylor *et al.* at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7. Consequently, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) and Taylor *et al.* (US Patent 5,001,225) fail to teach or suggest each and every element of claims 124 and 125.

In sum, Brandlein *et al.* (Amer. Assoc. Cancer Res. 43:970 abstract #4803 (2002)) alone or in combination with Taylor *et al.* (US Patent 5,001,225) fail to teach or suggest each and every element of claims 124 and 125, and fail to provide a reasonable expectation of success in producing an antibody or an antigen binding fragment at least 80% identical to the sequence of SEQ ID NO:5 or SEQ ID NO:7 of claims 124 and 125. As such, the rejection under 35 U.S.C. §103(a) is improper and Applicants respectfully request that the rejection be withdrawn.

CONCLUSION

In summary, for the reasons set forth herein, Applicants maintain that claims 111 to 134 clearly and patentably define the invention, respectfully request that the Examiner reconsider the various grounds set forth in the Office Action, and respectfully request the allowance of the claims which are now pending.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's representative can be reached at (858) 509-4065. Please charge any fees associated with the submission of this paper to Deposit Account Number 033975. The Commissioner for Patents is also authorized to credit any over payments to the above-referenced Deposit Account.

Respectfully submitted,

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Bedgood, Robert M.

From: Susan Hendriks [s.hendriks@iospress.nl]
Sent: Monday, July 09, 2007 4:28 AM
To: Bedgood, Robert M.
Subject: RE: Human Antibodies volume 11, number 4, 2002

Dear Robert,

Volume 11, number 4 of Human Anitbodies was published on April 18th 2003. I hope I've been of enough assistance in this matter. If you have any further questions, please do not hesitate to contact me.

Kind regards,

Susan Hendriks
Marketing Coordinator

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Van: Bedgood, Robert M. [mailto:robert.bedgood@pillsburylaw.com]
Verzonden: vrijdag 6 juli 2007 22:39
Aan: market@iospress.nl
Onderwerp: Human Antibodies volume 11, number 4, 2002

Dear Sir or Madam-
Please advise the month and date that Human Antibodies, Volume 11, Number 4, 2002 was published.
thanks you for your assistance.
regards,
Robert

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EXHIBIT A

Morningside | Translations

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TRANSLATOR CERTIFICATION

I, Michael Magee, a translator fluent in the German language, on behalf of Morningside Translations, do solemnly and sincerely declare that the following is, to the best of my knowledge and belief, a true and correct translation of the document(s) listed below in a form that best reflects the intention and meaning of the original text.

MORNINGSIDE TRANSLATIONS

Michael Magee
Signature of Translator

Date: August 27, 2007

Description of Documents Translated:

DE 102 29906A1

(19) Federal Republic of Germany
German Patent and Trademark Office

(10) **DE 102 29 906 A1** 01/22/2004

(12) # Unexamined Patent Application

(21) Application No.: **102 29 906.4**
(22) Filing Date: **July 4, 2002**
(43) Date laid open for public inspection: **January 22, 2004**

(51) Int. Cl.[7]: **C07K 16/00**
C12N 15/13

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(72) Inventor:
Same as applicant

(56) Documents cited in the evaluation of patentability:
DE 41 07 154 A1

The following information has been taken from documents filed by the applicant

Request for search in accordance with §43, Paragraph 1, Clause 1, German Patent Act has been filed.

(54) Title: **Human monoclonal antibody**

(57) Abstract: Described is a human monoclonal antibody containing heavy and light chain molecules, each having a region of constant composition from antibody to antibody and a region of variable composition from antibody to antibody. According to the invention, at least one variable region of the heavy chains has substantially the amino acid sequence stated in Appendix 1. The invention further relates to a method for producing the antibody, use of the antibody for combating tumors, and a pharmaceutical agent and diagnostic agent containing the antibody.

Description

[0001] The invention relates to a human monoclonal antibody containing heavy and light chain molecules, each having a region of constant composition from antibody to antibody and a region of variable composition from antibody to antibody, or a functional fragment thereof. The invention further relates to a method for producing the antibody, and use of the antibody for combating tumors, and a pharmaceutical agent and a diagnostic agent which contain the antibody.

[0002] Current methods for treating cancer include surgical removal of the tumor, radiation, and chemotherapy. A significant drawback of these methods is that they are not specifically targeted to the tumor cells. For surgical removal, for example, all of the tumor may not be removed, with the result that a new tumor develops and metastases possibly form which become established in other areas of the body. In the treatment of tumors by radiation or chemotherapeutic agents, the lack of selectivity often results in damage to healthy cells as well due to the agents used. The adverse result is that the doses of radiation or chemically active substances cannot be selected to be high enough that all the cancer cells are destroyed. A considerable portion of current cancer research is therefore devoted to finding more effective and in particular selectively active methods and agents for treating tumors.

[0003] Immunological studies have shown that cellular and humoral activity is measurable when the immune system cannot effectively combat malignant cells. However, this activity is not sufficient to destroy the tumor cells. A promising approach to combating tumors, therefore, is to isolate antibodies originating from the patient's immune response, suitably propagate the antibodies, and use them therapeutically.

[0004] One method from the prior art which uses this approach is the hybridoma technique, which is based on in vitro harvesting of cellular hybrids obtained by cellular fusion of normal lymphocytes with myeloma cells that are capable of unlimited viability and cell division. The hybridoma cells thus produced have the characteristics of both parent cells, and therefore have the ability of lymphocytes to produce antibodies, and also have the capability of myeloma cells for unlimited cell division and thus for producing antibodies in large quantities.

[0005] Each hybrid cell resulting from the fusion produces monoclonal antibodies whose specificity is determined by the original lymphocyte cell. The hybridoma cells are propagated, and the ones which produce antibodies of the desired specificity are then selected. Cultivation of this selection and isolation thereof results in highly specifically reactive antibodies which react only with a given antigenic determinant. Monoclonal antibodies which bind specifically to antigens of tumors thus offer promising opportunities for the diagnosis and treatment of tumor cells.

[0006] Thus, there is a need for such human monoclonal antibodies for improving the methods and agents for combating cancer. The object of the present invention is to provide a human monoclonal antibody, a method for production thereof, and diagnostic and pharmaceutical agents derived from the antibody which have a high specificity for antigens of various tumors, and which are therefore well suited for tumor-specific treatment and diagnosis.

[0007] This object is achieved according to the invention with regard to a human monoclonal antibody by the fact that

- at least one variable region of the light and/or of the heavy chains has substantially the amino acid sequence stated in Appendix 1.

[0008] From a chemical point of view, antibodies are immunoglobulin molecules. These molecules have two identical light chains and two identical heavy chains which are joined by disulfide bridges. Each of the chains has a region containing approximately 110 amino acids with a variable sequence, whereas the remainder of each chain has a region with a constant sequence. The variable regions of light and heavy chains each include multiple hypervariable regions which are responsible for binding of antigens. The specialized structure of the hypervariable regions thus determines the specific characteristics of the antibody.

[0009] Clinical tests have demonstrated that the structure of the referenced variable regions of the antibody according to the invention, depending on the given amino acid sequence, results in a high specific activity against the antigens of tumor cells under study. Since the antigens occurring on tumor cells are not present on normal cells, it is expected that the antibodies present exhibit little or no binding to normal cells.

[0010] It is essential to the invention that one of the variable regions of the light or heavy chains is substantially identical to the sequence according to the invention. By virtue of being substantially identical, the referenced regions predominantly match one another. The present invention encompasses minor modifications or substitutions of the chains, provided that the monoclonal antibody or the functional portion thereof maintains

tumor-specific characteristics.

[0011] The majority of tumor-specific monoclonal antibodies of the prior art involve antibodies derived from mice. However, it is disadvantageous that such antibodies have very limited use, since mouse antibodies used in humans are recognized by the immune system as foreign proteins and may be neutralized before their therapeutic effect can be realized.

[0012] In contrast, the invention proceeds from human monoclonal antibodies, which do not have these limitations of use in human medicine. These antibodies contain sequences in the hypervariable chain regions which substantially correspond to those of human immunoglobulin. The antibodies, after recognition of the determinants or epitopes of the corresponding antigens, are thus able to bind in an unhindered manner to the affected cells without a defense reaction of the immune system. When the antibodies according to the invention are coupled to diagnostic and therapeutic agents, such antibodies are thus advantageously suitable for early recognition and effective treatment of various types of tumors.

[0013] The object is achieved with regard to the production method by the fact that the human monoclonal antibody is preferably produced by the hybridoma technique. According to one feature of the invention, for this purpose B-lymphocytes are removed from a lymphatic organ, preferably the spleen or the lymph nodes, of a cancer patient. As a result of the cancer, these lymphocytes are stimulated to produce antibodies which react specifically to the antigens of the tumor cells that are present.

[0014] Each lymphocyte is fused with a myeloma cell in vitro. According to the present invention, HAB-1 heteromyeloma cells and their subclones are used. The HAB-1 heteromyeloma cell is described in the literature by Falter, G et al., HAB-1, BrJCancer 62, 595-8 (1990). Similarly, subclones of the HAB-1 cell, referred to as HAB-1.X, may be used. The resulting cell clones have the same characteristics as the original B-lymphocytes for producing antibodies. The specificity of these antibodies is determined by the original lymphocyte cell. In the present case, this means that the antibodies produced by the cell clones also correspond to the antigens of the specific tumor that is present. After the cells are selected which synthesize antibodies of the desired specificity, these cells are cultivated, and each of the hybrid cells produces human monoclonal antibody in unlimited quantities.

[0015] According to one feature of the invention, in the provided method lymphocytes are removed from patients in particular with carcinoma of the

- colon
- pancreas
- lung
- esophagus
- breast
- prostate

[0016] In addition to production of the present human monoclonal antibody by the hybridoma technique, the invention also encompasses other production methods. Direct synthesis by the recombinant method, known to one skilled in the art, or production using the known phage bank method (phage display) are provided, in particular for the production of small functional fragments.

[0017] Propagation is performed by use of the known polymerase chain reaction (PCR).

[0018] The PCR method is known to one skilled in the art, for example from US 4,683,195. This method is used for the targeted propagation of a specific DNA fragment, and is advantageously employed when DNA segments are present only in trace quantities. By use of the method, one known DNA sequence from a number of similar sequences may be identified, and may be propagated in vitro in large quantities in a short time. A specialized DNA sequence may be propagated by a factor of approximately 100,000 over a time period of approximately 3 h.

[0019] When the present method is used for producing the monoclonal antibodies according to the invention or a functional fragment thereof, RNA of the hybridoma cells, which produce tumor-specific monoclonal antibodies, is copied in vitro into complementary double-stranded cDNA, using reverse transcriptase. The cDNA, which codes functional fragments of the variable regions of the light and heavy chains, is then propagated using PCR. The PCR products are purified, extracted, and then cloned.

[0020] The composition of the constant region of the heavy chain of an antibody determines its isotype, and

establishes the effector function of the antibody. For immunoglobulin, the constant region of the heavy chains is composed of one of the sequences, referred to in the literature as μ, γ, δ, α, or ε, and the constant region of the light chains is composed of one of the sequences κ or λ. The various compositions of the heavy chains result in the five immunoglobulin classes IgA, IgD, IgE, IgG, and IgM. The antibodies according to the present invention generally belong to class IgM, and light chains of class λ and κ may be present. The antibody may also have a composition of the IgG class.

[0021] The invention encompasses human monoclonal antibodies and functional fragments thereof. The functionality of the referenced fragments is characterized in that said fragments have the characteristics of the antibody. Such characteristics, for example, may be that the fragments have the ability to bind with antigens, or have specificity for tumor cells, or that the fragments have an effector function due to the composition of their constant region. According to one feature of the invention, fragments in particular are included which according to known nomenclature (e.g., Cell Biophysics, 22 (1993), pp. 189–224) belong to one of the groups

V_L, V_H, Fv, Fc, Fab, Fab', $F(ab')_2$.

The V_L group comprises fragments which include the variable region, or the variable and constant region, of the light chains;

the V_H group comprises fragments which include the variable region, or the variable and constant region, of the heavy chains;

the Fv group comprises fragments which include the variable regions of the heavy and light chains or portions thereof;

the Fc group comprises fragments which include the constant regions of the heavy chains or portions thereof;

the Fab group comprises fragments which are larger than the fragments of the Fv group;

the Fab' group comprises fragments which are larger than the fragments of the Fab group;

the $F(ab')_2$ group comprises fragments which contain the variable regions of both heavy and both light chains or portions thereof, and which optionally contain the first constant regions of both heavy chains or portions thereof.

[0022] Specialized requirements for given applications may be met by use of the referenced fragments. According to one feature of the invention, the characteristics of the antibody or the functional fragments thereof may be modified by substituting and/or inserting and/or removing individual amino acid groups. These types of approaches may be used, for example, to modify the stability or the selectivity of the antibody or the functional fragments thereof while maintaining the global characteristics of the antibody, for example the ability to bind to tumor antigens.

[0023] According to the present invention, the antibodies or the functional fragments thereof may be joined to additional active substances. The fields of application for the present antibody may be significantly expanded by coupling of such substances. In particular, the human monoclonal antibody according to the present invention may be used for diagnostic methods for detecting tumor cells, and for therapeutic methods for combating tumor cells.

[0024] According to one feature of the invention, the following substances in particular are provided:

– a radioactive substance,
– and/or a dye,
– and/or an enzyme,
– and/or an immunotoxin,
– and/or a growth inhibitor,

[0025] whereby these active substances may be used for

– qualitative or quantitative detection,
– decreasing proliferation,
– producing apoptosis, or
– avoiding metastasis formation

[0026] of tumor cells.

[0027] The detection of tumor cells is frequently carried out using methods known by those skilled in the art as immunoassays, which are based on the antigen-antibody reaction. To obtain quantitative information from this reaction, the antibody according to the present invention is coupled to an easily detectable labeling substance. The substance and the coupling are selected such that the immunological characteristics of the components are substantially maintained. The most well-known immunoassays are the radioimmunoassay, enzyme immunoassay, and fluorescence immunoassay. The diagnostic substances coupled to the antibodies allow sensitive and reliable methods for early detection of cancer.

[0028] The purpose of the referenced cytotoxic substances, for example, is to reduce the viability or the division capability of the tumor cells. Alternatively, the substances suppress DNA synthesis or cell division, or cause apoptosis of the cells or nonapoptotic cell death, and thus terminate growth of tumor cells or kill such cells.
[0029] By coupling the referenced substances with antibodies according to the present invention that are selectively active against cancer cells, it is possible to combat various types of tumors in a targeted and particularly effective manner. The invention provides in particular for the
– diagnosis
– and/or prevention
– and/or treatment

[0030] of the following tumors:
– adenocarcinoma of the adrenal gland, uterus, pancreas, prostate,
– squamous cell carcinoma of the esophagus or lung,
– carcinoma of the stomach,
– ductal carcinoma of the breast.

[0031] Lastly, the present invention also encompasses a pharmaceutical agent and a diagnostic agent, characterized in that the active substances thereof contain the referenced monoclonal antibody or functional fragments thereof. The referenced agents generally contain further additives, such as physiological solutions, solvents, glycols, oils, or similar substances known from the prior art.

METHODS, EXAMPLES, AND PARTICULARS

[0032] PM-2 antibody
[0033] Heavy chain (VH)

Amino acid sequence

[0034] see Appendix 1

DNA sequence

[0035] see Appendix 1

Light chain (VL)

Amino acid sequence

[0036] see Appendix 2

DNA sequence

[0037] see Appendix 2

Method 1

Immortalization of lymphocytes and primary testing of antibodies

[0038] For immortalization, the lymphocytes were fused with a variant of HAB-1 heteromyeloma according to standard protocol and cultivated. In summary, lymphocytes were fused with HAB-1 cells by means of PEG. The trioma were seeded on four 24-hole plates. The average growth rate was 80-90%, with 50% of the grown clones secreting immunoglobulins.
[0039] The first testing of the secreted human monoclonal antibodies was carried out using ELISA to determine the isotype. The next test was an immunohistochemical staining on cryosections of the autologous tumor.

Media required

– RPMI 1640 (PAA) without additives

– RPMI 1640 containing HAT supplement (HAT supplement, PAA) and 10% FCS, 1% glutamine, and 1% penicillin/streptomycin

Immortalization

– Wash HAB-1 (fusion partner) twice with RPMI without additives
– Centrifuge for 5 min at 1500 rpm
– Thaw frozen lymphocytes (from spleen or lymph nodes) and wash twice with RPMI without additives, and likewise centrifuge
– Take up each of the two pellets in 10 mL RPMI without additives, and count in the Neubauer counting chamber
– Fuse in an HAB-1 to lymphocyte ratio of 1:2 to 1:3
– Combine the cell pellets after the second washing, mix, and centrifuge for 8 min at 1500 rpm
– Carefully add the PEG (polyethylene glycol 1500, Roche), previously warmed to 37°C, dropwise to the pellet with slight rotational motions of the 50-mL test tube
– Gently resuspend, and then allow to rotate for exactly 90 sec in a water bath at 37°C
– Wash out the PEG with RPMI without additives (two full No. 10 pipettes)
– Centrifuge for 5 min at 1500 rpm
– Plate 24-well plates with 1 mL RPMI containing HAT supplement
– Dissolve the pellet in RPMI containing HAT supplement
– Pipette 0.5 mL of the cells into each of the 24 wells
– Place fusion plates in drying oven
– Replace medium weekly with RPMI containing HAT supplement

Method 2

Molecular characterization of the antibodies by sequencing

[0040] For sequencing of the monoclonal antibodies, cDNA was produced from total RNA (RNAse kit, Quiagen) from trioma (M-MLV reverse transcriptase, Gibco). The corresponding VH genes were then propagated by PCR amplification (Taq polymerase, MBI Fermentas). The PCR products were purified by gel electrophoresis and extracted. After cloning of the PCR products (pCR-Script Amp SK + cloning kit, Stratagene), the positive closes were sequenced (DyeDeoxy termination cycle sequencing kit, Applied BioSystems). The sequences were analyzed using DNASIS for Windows, Genebank, and V-Base databases (Vollmers et al., 1998).

Immunohistochemical characterization

[0041] Antibodies which react with the autologous tumor were tested on a panel of normal tissues and tumorous tissues in the immunoperoxidase test (see below for protocol) in order to obtain an overview of the antibody reaction and the antigen distribution.
[0042] Antibodies which react specifically with the tumor cells but not with healthy tissue were tested further, first against the same types of tumors from different patients, then against tumors from other organs, and finally against normal tissue. More detailed characterization of the antibody and antigen is not possible unless the reaction pattern of the antibody supports a conclusion of at least limited specificity for malignant tissue.

Immunoperoxidase staining on cryosections and cytospins

– Slides
– Allow slides to dry for at least 2 h after cutting
– Place slides in acetone for 10 min
– Allow to dry for 30 min
– Wash 3x with Tris-NaCl and then allow to stand in Tris-NaCl for 5 min
– Saturate with 100 µL milk powder (3% in PBS) for 15–30 min
– Wash 3x with Tris-NaCl
– 100 µL of the respective 1st antibody:
– for negative control, with RPMI
– for positive control, 1:50 CK8 with BSA/PBS or 1:10 CAM 5.2 with BSA/PBS (0.5% BSA in PBS)
– Incubate for 30 min

– Wash 3x with Tris-NaCl
– 100 µL of the respective 2nd antibody:
– 70% conjugated rabbit anti-mouse peroxidase with PBS + 30% human serum + 1:50 antibody
– 70% conjugated rabbit anti-human IgM peroxidase with PBS + 30% rabbit serum + 1:50 antibody
– Incubate for 30 min
– Wash 3x with Tris-NaCl
– Place slides in PBS for 10 min
– Dissolve 1 DAB tablet and 1 H_2O_2 tablet in 1 mL tap water
– Pipette 100 µL substrate onto the slides and incubate for 10 min
– Rinse with distilled water
– Place slides in hemalum for 5 min
– Rinse with water for 15 min
– Place slides in distilled water and mount with glycerin gelatin

IMMUNOPEROXIDASE STAINING ON PARAFFIN SECTIONS

Deparaffination
– Xylene 1x, 5 min
– Xylene 2x, 5 min
– 100% ethanol 1x, 5 min
– 100% ethanol 2x, 5 min
– Methanol (70 mL) + H_2O_2 (500 µL), 5 min
– 90% ethanol 1x, 3 min
– 90% ethanol 2x, 3 min
– 80% ethanol 1x, 3 min
– 80% ethanol 2x, 3 min
– 70% ethanol 1x, 3 min
– 70% ethanol 2x, 3 min

[0043] Wash 1x with Tris-NaCl
Boil 300 mL distilled H_2O in pressure cooker and fill citric acid (pH 5.5) into insert, boil slides for 5 min
Block for 15 min with BSA/PBS, 150 µL per slide
Wash 1x with Tris-NaCl
Incubate 1st antibody, 150 µL per slide, for 2.5 h in a humidified chamber in drying oven
Wash 3x with Tris-NaCl
Incubate 2nd antibody, 150 µL per slide, for 45 min in a humidified chamber at room temperature
Wash 3x with Tris-NaCl
Allow to stand for 10 min in PBS
10 min DAB, 150 µL per slide, wash 3x with H_2O, then wash 1x with distilled H_2O
Stain for 5 min with hemalum
Rinse with water for 10–15 min
Wash with distilled H_2O
Mount with glycerin gelatin

Staining of tumorous tissue

[0044] The tumorous tissues were stained to evaluate the number of carcinomas to which the antibody being studied showed a reaction.

Staining of normal tissue

PM-1

Tissue	CAM5.2	PM-1	M6 (IgM control)
Pancreas	–	–	–
Breast	–	–	–
Stomach	–	–	–
Prostate	–	–	–
Colon	–	–	–
Fallopian tube	–	–	–
Esophagus	–	–	–
Bladder	–	–	–
Small intestine	–	–	–

Staining of tumorous tissue

Tumor	CAM5.2	PM-1	M6 (IgM control)
Lung (squamous cell)	+ (CK5/6)	+	–
Lung (adeno)	+	–	–
Breast	+	+	–
Prostate	+	+	–
Adrenal gland	+	+	–
Liver	+	–	–
Bladder	+	–	–
Small intestine	+	+	–
Colon	+	+	
Stomach	+	+	
Esophagus	+ (CK5/6)	+	
Pancreas	+	+	
Uterus	+	+	

Cell Death ELISA^{PLUS} (Roche, Mannheim)

[0045] The extent of apoptosis induction by CM-1 antibody was analyzed using Cell Death Detection ELISA^{PLUS}. This test is based on the principle of a quantitative sandwich enzyme immunoassay, using the peroxidase-conjugated mouse monoclonal antibodies directed against histone or DNA components. Following enzymatic conversion of a colorless substrate, the quantity of nucleosomes, and thus the relative number of apoptotic cells, present may be photometrically determined on the basis of the color intensity of the reaction product.

[0046] For this purpose, 100 µL of a cell suspension (1.0 x 10^5 /mL) of the various cell lines together with 100 µL of the undiluted or 1:1 diluted antibody supernatants in a 96-well plate were incubated for 24 h in a drying oven at 37°C and 7% CO_2. After completion of incubation the cells were centrifuged for 10 min at 200 g, the supernatant was aspirated, and 200 µL lysis buffer was added, resulting in cell lysis over the following 30 min at room temperature. After recentrifugation, 20 µL of the supernatant was transferred to each streptavidin-coated microtiter plate, and 80 µL of the immunoreagent (1/20 anti-DNA-POD, 1/20 anti-histone biotin, 18/20 incubation buffer) was then added by pipette. In addition, a positive control contained in the test kit and a blank assay were performed. After thorough mixing of the plates for 2 hours at approximately 250 rpm, followed by washing three times with incubation buffer (250 µL), 100 µL of the ABTS solution (1 ABTS tablet in 5 mL substrate buffer) was pipetted into each well. After remixing, the intensity of the antibody-induced apoptosis was indicated by an intense green precipitate. The color intensity was measured using an ELISA reader at λ =

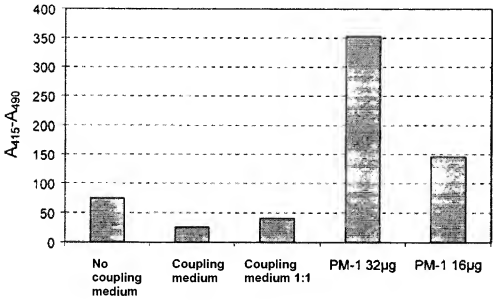
415 nm against a reference wavelength of 490 nm, and on this basis the intensity of the antibody-induced apoptosis was calculated.

Cell Death ELISA

Antibody: PM-1

Cell line: BXPC-3

Incubation time: 24 h



Without: Negative control (RPMI 1460 medium)
PM-1: 16 or 32 µg/mL antibody supernatant

0048] After incubation for 24 hours, the PM-2 antibody being studied showed pronounced apoptosis indication [sic; induction] compared to the negative controls.

MTT test

– Trypsinize cells and resuspend in 10 mL RPMI complete medium (RPMI 1640, 10% FCS, 1% glutamine, 1% penicillin/streptomycin)
– Count cells and dilute to 1 x 10^6 cells per mL
– Pipette 50 µL cell suspension per well into a 96-well plate (leave first row empty); i.e., a cell count of 5 x 10^4 cells is present in each well
– Add 50 µL antibody (various dilutions in complete medium) per well
– Incubate the 96-well plate for 24 h or 48 h in a drying oven
– Pipette 50 µL MTT solution into each well
– Incubate plate for 20 min in the drying oven
– Centrifuge plate for 10 min at 2800 rpm and aspirate supernatant
– Add 150 µL DMSO per well and resuspend the cell pellet
– Determine absorption at a wavelength of 540 nm and 690 nm, using the ELISA reader.

[0049] MTT: Dissolve 5 mg/mL 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium (SIGMA) in PBS.

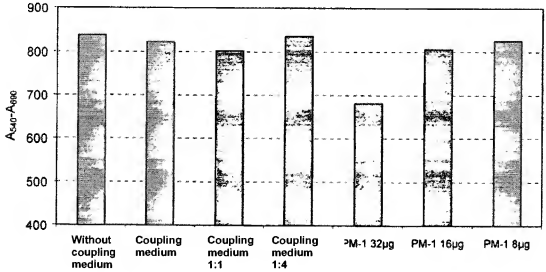
[0050]

MTT test

Antibody: **PM-1**

Cell line:BXPC-3 (carcinoma of the pancreas)

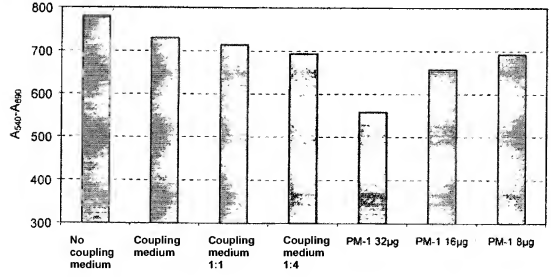
Incubation time: 24 h



Antibody: **PM-1**

Cell line:BXPC-3 (carcinoma of the pancreas)

Incubation time: 48 h



<110> Prof. Dr. Müller-Hermelink, Hans Konrad
Prof. Dr. Vollmers, H. Peter

<120> Human monoclonal antibody

<141> May 13, 2002

<211> 294

<212> DNA

<213> Homo sapiens

<220> Sequence of the variable region of the heavy chain (V_H) of PM-1 antibody (clone 7/99-38)

<221> V region

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ggg tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc ttt agc agc tat gcc atg agc      60
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Ala Met Ser
 1               5                  10                  15                  20

tgg gtc cgc cag gct cca ggg aag ggg ctg gag tgg gtc tca gct att agt ggt agt ggt     120
Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ser Ala Ile Ser Gly Ser Gly
            25                  30                  35                  40

ggt agc aca tac tac gca gac tcc gtg aag ggc cgg ttc acc atc tcc aga gac aat tcc     180
Gly Ser Thr Tyr Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
            45                  50                  55                  60

aag aac acg ctg tat ctg caa atg aac agc ctg aga gcc gag gac acg gcc gta tat tac     240
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr
            65                  70                  75                  80

tgt gcg aaa gat tca ttt cgt gaa gga ccc tgg ggc cag gga acc ctg gtc acc           294
Cys Ala Lys Asp Ser Phe Arg Glu Gly Pro Trp Gly Gln Gly Thr Leu Val Thr
                85                  90                  95
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<110> Prof. Dr. Müller-Hermelink, Hans Konrad
Prof. Dr. Vollmers, H. Peter

<120> Human monoclonal antibody

<141> May 13, 2002

<211> 318

<212> DNA

<213> Homo sapiens

<220> Sequence of the variable region of the heavy chain (V_H) of PM-1 antibody (clone 7/99-38)

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Ser Tyr Val Leu Thr Gln Pro Pro Ser Val Ser Val Ser Pro Gly Gln Thr Ala Arg Ile
 1               5                  10                  15                  20

acc tgc tct gga gat gca ttg cca aaa aaa tat gct tat tgg tac cag cag aag tca ggc      120
Thr Cys Ser Gly Asp Ala Leu Pro Lys Lys Tyr Ala Tyr Trp Tyr Gln Gln Lys Ser Gly
                25                  30                  35                  40

cag gcc cct gtg ctg gtc atc tat gag gac agc aaa cga ccc tcc ggg atc cct gag aga      180
Gln Ala Pro Val Leu Val Ile Tyr Glu Asp Ser Lys Arg Pro Ser Gly Ile Pro Glu Arg
                45                  50                  55                  60

ttc tct ggc tcc agc tca ggg aca atg gcc acc ttg act atc agt ggg gcc cag gtg gag      240
Phe Ser Gly Ser Ser Ser Gly Thr Met Ala Thr Leu Thr Ile Ser Gly Ala Gln Val Glu
                65                  70                  75                  80

gat gaa gct gac tac tat tgt tac tca aca gac agc agt ggt aat atg tct tcg gaa ctg      300
Asp Glu Ala Asp Tyr Tyr Cys Tyr Ser Thr Asp Ser Ser Gly Asn Met Ser Ser Glu Leu
                85                  90                  95                  100

gga cca agc tca ccg tcc                                                          318
Gly Pro Ser Ser Pro Ser
                105
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Claims

1. Human monoclonal antibody containing heavy and light chain molecules, each having a region of constant composition from antibody to antibody and a region of variable composition from antibody to antibody, or a functional fragment thereof, **characterized in that**
at least one variable region of the light chains has substantially the amino acid sequence stated in Appendix 2, and/or at least one variable region of the heavy chains has substantially the amino acid sequence stated in Appendix 1.

2. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 1 using the hybridoma technique, characterized in that

– the hybridoma cells are obtained by fusion
– of HAB-1 heteromyeloma cells and their subclones
– with B-lymphocytes removed from
– a lymphatic organ, preferably the spleen or the lymph nodes, of a cancer patient.

3. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 2, characterized in that
– the B-lymphocytes are removed from a patient with
– carcinoma of the colon, pancreas, lung, esophagus, breast, or prostate.

4. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 1, characterized in that
– the human monoclonal antibody or fragment thereof is produced using the recombinant method.

5. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 1, characterized in that
– the human monoclonal antibody or fragment thereof is produced by gene technology,
– using phage banks (phage display method).

6. Human monoclonal antibody or a functional fragment thereof according to one of Claims 1–5, characterized in that
– the structure of the constant region of the heavy chains corresponds to immunoglobulin M or G (IgM or IgG).

7. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–6, characterized in that
the referenced functional fragment belongs to one of the groups
– V_L
– V_H
– Fv
– Fc
– Fab
– Fab'
– $F(ab')_2$.

8. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–7, characterized in that
– individual amino acid groups are
– substituted,
– and/or inserted,
– and/or removed.

9. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–8, characterized in that
– a first substance is coupled, in particular
– a radioactive substance,
– and/or a dye,
– and/or an enzyme,
– and/or an immunotoxin,
– and/or a growth inhibitor.

10. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–9, characterized in that
– a second substance is coupled, in particular for
– qualitative or quantitative detection,
– decreasing proliferation,
– producing apoptosis,
– avoiding metastasis formation of tumor cells.

11. Use of the monoclonal antibody or a functional fragment thereof according to one of the preceding claims for combating tumors, characterized in that the monoclonal antibody or functional fragment thereof is used for the
– diagnosis
– and/or prevention
– and/or treatment of the following tumors in particular:
adenocarcinoma of the adrenal gland, uterus, pancreas, or prostate,
squamous cell carcinoma of the esophagus or lung,
carcinoma of the stomach,
ductal carcinoma of the breast.

12. Pharmaceutical agent, characterized in that
– the active substance thereof contains the referenced monoclonal antibody or functional fragments thereof.

13. Diagnostic agent, characterized in that
– the active substance thereof contains the referenced monoclonal antibody or functional fragments thereof.

No drawing sheet is appended

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TRANSLATOR CERTIFICATION

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I, Michael Magee, a translator fluent in the German language, on behalf of Morningside Translations, do solemnly and sincerely declare that the following is, to the best of my knowledge and belief, a true and correct translation of the document(s) listed below in a form that best reflects the intention and meaning of the original text.

MORNINGSIDE TRANSLATIONS

Michael Magee | amp

Signature of Translator

Date: August 27, 2007

Description of Documents Translated:

DE 102 29907A1

(19) Federal Republic of Germany
German Patent and Trademark Office

(10) **DE 102 29 907 A1** 01/22/2004

(12) # Unexamined Patent Application

(21) Application No.: **102 29 907.2**
(22) Filing Date: **July 4, 2002**
(43) Date laid open for public inspection: **January 22, 2004**

(51) Int. Cl.[7]: **C07K 16/00**
A61K 39/395

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(56) Documents cited in the evaluation of patentability:
DE 41 07 154 A1

The following information has been taken from documents filed by the applicant

Request for search in accordance with §43, Paragraph 1, Clause 1, German Patent Act has been filed.

(54) Title: **Human monoclonal antibody**

(57) Abstract: Described is a human monoclonal antibody containing heavy and light chain molecules, each having a region of constant composition from antibody to antibody and a region of variable composition from antibody to antibody. According to the invention, at least one variable region of the heavy chains has substantially the amino acid sequence stated in Appendix 1. The invention further relates to a method for producing the antibody, use of the antibody for combating tumors, and a pharmaceutical agent and diagnostic agent containing the antibody.

Description

[0001] The invention relates to a human monoclonal antibody containing heavy and light chain molecules, each having a region of constant composition from antibody to antibody and a region of variable composition from antibody to antibody, or a functional fragment thereof. The invention further relates to a method for producing the antibody, and use of the antibody for combating tumors, and a pharmaceutical agent and a diagnostic agent which contain the antibody.

[0002] Current methods for treating cancer include surgical removal of the tumor, radiation, and chemotherapy. A significant drawback of these methods is that they are not specifically targeted to the tumor cells. For surgical removal, for example, all of the tumor may not be removed, with the result that a new tumor develops and metastases possibly form which become established in other areas of the body. In the treatment of tumors by radiation or chemotherapeutic agents, the lack of selectivity often results in damage to healthy cells as well due to the agents used. The adverse result is that the doses of radiation or chemically active substances cannot be selected to be high enough that all the cancer cells are destroyed. A considerable portion of current cancer research is therefore devoted to finding more effective and in particular selectively active methods and agents for treating tumors.

[0003] Immunological studies have shown that cellular and humoral activity is measurable when the immune system cannot effectively combat malignant cells. However, this activity is not sufficient to destroy the tumor cells. A promising approach to combating tumors, therefore, is to isolate antibodies originating from the patient's immune response, suitably propagate the antibodies, and use them therapeutically.

[0004] One method from the prior art which uses this approach is the hybridoma technique, which is based on in vitro harvesting of cellular hybrids obtained by cellular fusion of normal lymphocytes with myeloma cells that are capable of unlimited viability and cell division. The hybridoma cells thus produced have the characteristics of both parent cells, and therefore have the ability of lymphocytes to produce antibodies, and also have the capability of myeloma cells for unlimited cell division and thus for producing antibodies in large quantities.

[0005] Each hybrid cell resulting from the fusion produces monoclonal antibodies whose specificity is determined by the original lymphocyte cell. The hybridoma cells are propagated, and the ones which produce antibodies of the desired specificity are then selected. Cultivation of this selection and isolation thereof results in highly specifically reactive antibodies which react only with a given antigenic determinant. Monoclonal antibodies which bind specifically to antigens of tumors thus offer promising opportunities for the diagnosis and treatment of tumor cells.

[0006] Thus, there is a need for such human monoclonal antibodies for improving the methods and agents for combating cancer. The object of the present invention is to provide a human monoclonal antibody, a method for production thereof, and diagnostic and pharmaceutical agents derived from the antibody which have a high specificity for antigens of various tumors, and which are therefore well suited for tumor-specific treatment and diagnosis.

[0007] This object is achieved according to the invention with regard to a human monoclonal antibody by the fact that

- at least one variable region of the light chains has substantially the amino acid sequence stated in Appendix 2, and/or at least one variable region of the heavy chains has substantially the amino acid sequence stated in Appendix 1.

[0008] From a chemical point of view, antibodies are immunoglobulin molecules. These molecules have two identical light chains and two identical heavy chains which are joined by disulfide bridges. Each of the chains has a region containing approximately 110 amino acids with a variable sequence, whereas the remainder of each chain has a region with a constant sequence. The variable regions of light and heavy chains each include multiple hypervariable regions which are responsible for binding of antigens. The specialized structure of the hypervariable regions thus determines the specific characteristics of the antibody.

[0009] Clinical tests have demonstrated that the structure of the referenced variable regions of the antibody according to the invention, depending on the given amino acid sequence, results in a high specific activity against the antigens of tumor cells under study. Since the antigens occurring on tumor cells are not present on normal cells, it is expected that the antibodies present exhibit little or no binding to normal cells.

[0010] It is essential to the invention that one of the variable regions of the light or heavy chains is substantially identical to the sequence according to the invention. By virtue of being substantially identical, the referenced regions predominantly match one another. The present invention encompasses minor modifications or substitutions of the chains, provided that the monoclonal antibody or the functional portion thereof maintains

tumor-specific characteristics.

[0011] The majority of tumor-specific monoclonal antibodies of the prior art involve antibodies derived from mice. However, it is disadvantageous that such antibodies have very limited use, since mouse antibodies used in humans are recognized by the immune system as foreign proteins and may be neutralized before their therapeutic effect can be realized.

[0012] In contrast, the invention proceeds from human monoclonal antibodies, which do not have these limitations of use in human medicine. These antibodies contain sequences in the hypervariable chain regions which substantially correspond to those of human immunoglobulin. The antibodies, after recognition of the determinants or epitopes of the corresponding antigens, are thus able to bind in an unhindered manner to the affected cells without a defense reaction of the immune system. When the antibodies according to the invention are coupled to diagnostic and therapeutic agents, such antibodies are thus advantageously suitable for early recognition and effective treatment of various types of tumors.

[0013] The object is achieved with regard to the production method by the fact that the human monoclonal antibody is preferably produced by the hybridoma technique. According to one feature of the invention, for this purpose B-lymphocytes are removed from a lymphatic organ, preferably the spleen or the lymph nodes, of a cancer patient. As a result of the cancer, these lymphocytes are stimulated to produce antibodies which react specifically to the antigens of the tumor cells that are present.

[0014] Each lymphocyte is fused with a myeloma cell in vitro. According to the present invention, HAB-1 heteromyeloma cells and their subclones are used. The HAB-1 heteromyeloma cell is described in the literature by Falter, G et al., HAB-1, BrJCancer 62, 595-8 (1990). Similarly, subclones of the HAB-1 cell, referred to as HAB-1.X, may be used. The resulting cell clones have the same characteristics as the original B-lymphocytes for producing antibodies. The specificity of these antibodies is determined by the original lymphocyte cell. In the present case, this means that the antibodies produced by the cell clones also correspond to the antigens of the specific tumor that is present. After the cells are selected which synthesize antibodies of the desired specificity, these cells are cultivated, and each of the hybrid cells produces human monoclonal antibody in unlimited quantities.

[0015] According to one feature of the invention, in the provided method lymphocytes are removed from patients in particular with carcinoma of the

- stomach
- colon
- pancreas
- esophagus
- lung
- prostate
- breast.

[0016] In addition to production of the present human monoclonal antibody by the hybridoma technique, the invention also encompasses other production methods. Direct synthesis by the recombinant method, known to one skilled in the art, or production using the known phage bank method (phage display) are provided, in particular for the production of small functional fragments.

[0017] Propagation is performed by use of the known polymerase chain reaction (PCR).

[0018] The PCR method is known to one skilled in the art, for example from US 4,683,195. This method is used for the targeted propagation of a specific DNA fragment, and is advantageously employed when DNA segments are present only in trace quantities. By use of the method, one known DNA sequence from a number of similar sequences may be identified, and may be propagated in vitro in large quantities in a short time. A specialized DNA sequence may be propagated by a factor of approximately 100,000 over a time period of approximately 3 h.

[0019] When the present method is used for producing the monoclonal antibodies according to the invention or a functional fragment thereof, RNA of the hybridoma cells, which produce tumor-specific monoclonal antibodies, is copied in vitro into complementary double-stranded cDNA, using reverse transcriptase. The cDNA, which codes functional fragments of the variable regions of the light and heavy chains, is then propagated using PCR. The PCR products are purified, extracted, and then cloned.

[0020] The composition of the constant region of the heavy chain of an antibody determines its isotype, and establishes the effector function of the antibody. For immunoglobulin, the constant region of the heavy chains is composed of one of the sequences, referred to in the literature as μ, γ, δ, α, or ε, and the constant region of the light chains is composed of one of the sequences κ or λ. The various compositions of the heavy chains result in the five immunoglobulin classes IgA, IgD, IgE, IgG, and IgM. The antibodies according to the present invention generally belong to class IgM, and light chains of class λ and κ may be present. The antibody may also have a composition of the IgG class.

[0021] The invention encompasses human monoclonal antibodies and functional fragments thereof. The functionality of the referenced fragments is characterized in that said fragments have the characteristics of the antibody. Such characteristics, for example, may be that the fragments have the ability to bind with antigens, or have specificity for tumor cells, or that the fragments have an effector function due to the composition of their constant region. According to one feature of the invention, fragments in particular are included which according to known nomenclature (e.g., Cell Biophysics, 22 (1993), pp. 189–224) belong to one of the groups
V_L, V_H, Fv, Fc, Fab, Fab', $F(ab')_2$.
The V_L group comprises fragments which include the variable region, or the variable and constant region, of the light chains;
the V_H group comprises fragments which include the variable region, or the variable and constant region, of the heavy chains;
the Fv group comprises fragments which include the variable regions of the heavy and light chains or portions thereof;
the Fc group comprises fragments which include the constant regions of the heavy chains or portions thereof;
the Fab group comprises fragments which are larger than the fragments of the Fv group;
the Fab' group comprises fragments which are larger than the fragments of the Fab group;
the $F(ab')_2$ group comprises fragments which contain the variable regions of both heavy and both light chains or portions thereof, and which optionally contain the first constant regions of both heavy chains or portions thereof.

[0022] Specialized requirements for given applications may be met by use of the referenced fragments. According to one feature of the invention, the characteristics of the antibody or the functional fragments thereof may be modified by substituting and/or inserting and/or removing individual amino acid groups. These types of approaches may be used, for example, to modify the stability or the selectivity of the antibody or the functional fragments thereof while maintaining the global characteristics of the antibody, for example the ability to bind to tumor antigens.

[0023] According to the present invention, the antibodies or the functional fragments thereof may be joined to additional active substances. The fields of application for the present antibody may be significantly expanded by coupling of such substances. In particular, the human monoclonal antibody according to the present invention may be used for diagnostic methods for detecting tumor cells, and for therapeutic methods for combating tumor cells.

[0024] According to one feature of the invention, the following substances in particular are provided:
– a radioactive substance,
– and/or a dye,
– and/or an enzyme,
– and/or an immunotoxin,
– and/or a growth inhibitor,

whereby these active substances may be used for
– qualitative or quantitative detection,
– decreasing proliferation,
– producing apoptosis, or
– avoiding metastasis formation
of tumor cells.

[0025] The detection of tumor cells is frequently carried out using methods known by those skilled in the art as immunoassays, which are based on the antigen-antibody reaction. To obtain quantitative information from this reaction, the antibody according to the present invention is coupled to an easily detectable labeling substance. The substance and the coupling are selected such that the immunological characteristics of the components are substantially maintained. The most well-known immunoassays are the radioimmunoassay, enzyme immunoassay, and fluorescence immunoassay. The diagnostic substances coupled to the antibodies allow sensitive and reliable methods for early detection of cancer.

[0026] The purpose of the referenced cytotoxic substances, for example, is to reduce the viability or the division capability of the tumor cells. Alternatively, the substances suppress DNA synthesis or cell division, or cause apoptosis of the cells or nonapoptotic cell death, and thus terminate growth of tumor cells or kill such cells.
[0027] By coupling the referenced substances with antibodies according to the present invention that are selectively active against cancer cells, it is possible to combat various types of tumors in a targeted and particularly effective manner. The invention provides in particular for the
– diagnosis
– and/or prevention
– and/or treatment

of the following tumors:
– adenocarcinoma of the colon,
– adenocarcinoma of the endometrium.

[0028] Lastly, the present invention also encompasses a pharmaceutical agent and a diagnostic agent, characterized in that the active substances thereof contain the referenced monoclonal antibody or functional fragments thereof. The referenced agents generally contain further additives, such as physiological solutions, solvents, glycols, oils, or similar substances known from the prior art.

METHODS, EXAMPLES, AND PARTICULARS

CM-2 antibody

Heavy chain (VH)

[09] Amino acid sequence
see Appendix 1
DNA sequence
see Appendix 1

Light chain (VL)

[0030] Amino acid sequence
see Appendix 2
DNA sequence
see Appendix 2

Method 1:

Immortalization of lymphocytes and primary testing of antibodies

[0031] For immortalization, the lymphocytes were fused with a variant of HAB-1 heteromyeloma according to standard protocol and cultivated. In summary, lymphocytes were fused with HAB-1 cells by means of PEG. The trioma were seeded on four 24-hole plates. The average growth rate was 80-90%, with 50% of the grown clones secreting immunoglobulins.
[0032] The first testing of the secreted human monoclonal antibodies was carried out using ELISA to determine the isotype. The next test was an immunohistochemical staining on cryosections of the autologous tumor.

Media required:

– RPMI 1640 (PAA) without additives
– RPMI 1640 containing HAT supplement (HAT supplement, PAA) and 10% FCS, 1% glutamine, and 1% penicillin/streptomycin

Immortalization:

– Wash HAB-1 (fusion partner) twice with RPMI without additives
– Centrifuge for 5 min at 1500 rpm

– Thaw frozen lymphocytes (from spleen or lymph nodes) and wash twice with RPMI without additives, and likewise centrifuge
– Take up each of the two pellets in 10 mL RPMI without additives, and count in the Neubauer counting chamber
– Fuse in an HAB-1 to lymphocyte ratio of 1:2 to 1:3
– Combine the cell pellets after the second washing, mix, and centrifuge for 8 min at 1500 rpm
– Carefully add the PEG (polyethylene glycol 1500, Roche), previously warmed to 37°C, dropwise to the pellet with slight rotational motions of the 50-mL test tube
– Gently resuspend, and then allow to rotate for exactly 90 sec in a water bath at 37°C
– Wash out the PEG with RPMI without additives (two full No. 10 pipettes)
– Centrifuge for 5 min at 1500 rpm
– Plate 24-well plates with 1 mL RPMI containing HAT supplement
– Dissolve the pellet in RPMI containing HAT supplement
– Pipette 0.5 mL of the cells into each of the 24 wells
– Place fusion plates in drying oven
– Replace medium weekly with RPMI containing HAT supplement

Method 2:

[0033]

Molecular characterization of the antibodies

[0034] For sequencing of the monoclonal antibodies, cDNA was produced from total RNA (RNAse kit, Quiagen) from trioma (M-MLV reverse transcriptase, Gibco). The corresponding VH genes were then propagated by PCR amplification (Taq polymerase, MBI Fermentas). The PCR products were purified by gel electrophoresis and extracted. After cloning of the PCR products (pCR-Script Amp SK + cloning kit, Stratagene), the positive closes were sequenced (DyeDeoxy termination cycle sequencing kit, Applied BioSystems). The sequences were analyzed using DNASIS for Windows, Genebank, and V-Base databases (Vollmers et al., 1998).

Immunohistochemical characterization

[0035] Antibodies which react with the autologous tumor were tested on a panel of normal tissues and tumorous tissues in the immunoperoxidase test (see below for protocol) in order to obtain an overview of the antibody reaction and the antigen distribution.
[0036] Antibodies which react specifically with the tumor cells but not with healthy tissue were tested further, first against the same types of tumors from different patients, then against tumors from other organs, and finally against normal tissue. More detailed characterization of the antibody and antigen is not possible unless the reaction pattern of the antibody supports a conclusion of at least limited specificity for malignant tissue.

Immunoperoxidase staining on cryosections and cytospins

– Slides
– Allow slides to dry for at least 2 h after cutting
– Place slides in acetone for 10 min
– Allow to dry for 30 min
– Wash 3x with Tris-NaCl and then allow to stand in Tris-NaCl for 5 min
– Saturate with 100 µL milk powder (3% in PBS) for 15–30 min
– Wash 3x with Tris-NaCl
– 100 µL of the respective 1st antibody:
– for negative control, with RPMI
– for positive control, 1:50 CK8 with BSA/PBS or 1:10 CAM 5.2 with BSA/PBS (0.5% BSA in PBS)
– Incubate for 30 min
– Wash 3x with Tris-NaCl
– 100 µL of the respective 2nd antibody:
– 70% conjugated rabbit anti-mouse peroxidase with PBS + 30% human serum + 1:50 antibody
– 70% conjugated rabbit anti-human IgM peroxidase with PBS + 30% rabbit serum + 1:50 antibody
– Incubate for 30 min

– Wash 3x with Tris-NaCl
– Place slides in PBS for 10 min
– Dissolve 1 DAB tablet and 1 H_2O_2 tablet in 1 mL tap water
– Pipette 100 µL substrate onto the slides and incubate for 10 min
– Rinse with distilled water
– Place slides in hemalum for 5 min
– Rinse with water for 15 min
– Place slides in distilled water and mount with glycerin gelatin

Staining of tumorous tissue

[0037] Tumorous tissues were stained to evaluate the number of carcinomas to which the antibody being studied showed a reaction.

Staining of tumorous tissue

Type of tumor	CM-2
Colon, adenocarcinoma	+
Thyroid, carcinoma	–
Bronchial carcinoma, squamous cell	–
Bronchial carcinoma, small cell	–
Bronchial carcinoma, large cell	–
Bronchial carcinoma, carcinoid	–
Stomach, adenocarcinoma	–
Esophagus carcinoma, squamous cell	–
Pancreas, adenocarcinoma	–
Hepatocell, carcinoma	–
Cholangiocell, carcinoma	–

Kidney cell, carcinoma	–
Nephroblastoma	–
Bladder, urothelial carcinoma	–
Breast carcinoma, ductal	–
Breast carcinoma, lobular	+
Endometrium, adenocarcinoma	–
Prostate, adenocarcinoma	–
Seminoma	–
Umbilical vesicle tumor	–
Teratoma	–
Teratocarcinoma	–
Melanoma	–
Thymoma	–
Fibrosarcoma	–
Myxofibrosarcoma	–
Rhabdomyosarcoma	–
Leiomyosarcoma	–
Neuroblastoma	–
Oropharyngeal carcinoma, squamous cell	–
Acute myeloid leukemia	–

Staining of normal tissue

Organ	CM-2	Organ	CM-2
Thyroid	–	Pineal gland	–
Lung	–	Pituitary gland	–
Aorta	–	Bone marrow	–
Myocardium	–	Blood	–
Pericardium	–	Cerebellum	–
Tongue	–		
Esophagus	–		
Stomach	–		
Small intestine	–		
Colon	–		

Rectum	–
Pancreas	–
Thymus	–
Tonsil	–
Lymph nodes	–
Adrenal gland	–
Kidney	–
Bladder	–
Vas deferens	–
Prostate	–
Testicle	–
Breast	–
Ovary	–
Fallopian tube	–
Uterus	–
Uterine cervix, portio vaginalis	–
Skin	–
Skeletal muscle	–
Placenta	–
Spinal medulla	–
Cerebral cortex	–

Staining of fetal tissue

Organ	CM-2
Lung	–
Stomach	–
Ileum	–
Pancreas	–
Liver	–
Spleen	–
Thymus	–
Kidney	–
Spinal medulla	–
Cerebral cortex	–
Cerebellum	–
Pineal gland	–
Pituitary gland	–

Cell Death ELISA^{PLUS} (Roche, Mannheim)

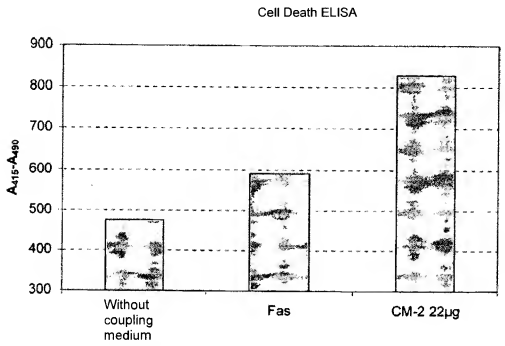
[0038] The extent of apoptosis induction by CM-1 antibody was analyzed using Cell Death Detection ELISA^{PLUS}. This test is based on the principle of a quantitative sandwich enzyme immunoassay, using the peroxidase-conjugated mouse monoclonal antibodies directed against histone or DNA components. Following enzymatic conversion of a colorless substrate, the quantity of nucleosomes, and thus the relative number of apoptotic cells, present may be photometrically determined on the basis of the color intensity of the reaction product.

[0039] For this purpose, 100 µL of a cell suspension (1.0 x 10^5 /mL) of the various cell lines together with 100 µL of the undiluted or 1:1 diluted antibody supernatants in a 96-well plate were incubated for 24 h in a drying oven at 37°C and 7% CO_2. After completion of incubation the cells were centrifuged for 10 min at 200 g, the supernatant was aspirated, and 200 µL lysis buffer was added, resulting in cell lysis over the following 30 min at room temperature. After recentrifugation, 20 µL of the supernatant was transferred to each streptavidin-coated microtiter plate, and 80 µL of the immunoreagent (1/20 anti-DNA-POD, 1/20 anti-histone biotin, 18/20 incubation buffer) was then added by pipette. In addition, a positive control contained in the test kit and a blank assay were performed. After thorough mixing of the plates for 2 hours at approximately 250 rpm, followed by washing three times with incubation buffer (250 µL), 100 µL of the ABTS solution (1 ABTS tablet in 5 mL substrate buffer) was pipetted into each well. After remixing, the intensity of the antibody-induced apoptosis was indicated by an intense green precipitate. The color intensity was measured using an ELISA reader at λ = 415 nm against a reference wavelength of 490 nm, and on this basis the intensity of the antibody-induced apoptosis was calculated.

[0040]

Antibody: CM-2
Cell line: CACO-2
Incubation time: 24 h

[0041] After incubation for 24 hours, the CM-2 antibody being studied showed pronounced apoptosis indication [sic; induction] compared to the negative controls, the effect for CM-2 exceeding that of the negative control by a factor of 1.46.



Without:	Negative control (RPMI 1460 medium)	
CD95 Fas:	2 µg/mL	Positive control (commercially available antibody)
CM-2:	22 µg/mL	Antibody supernatant

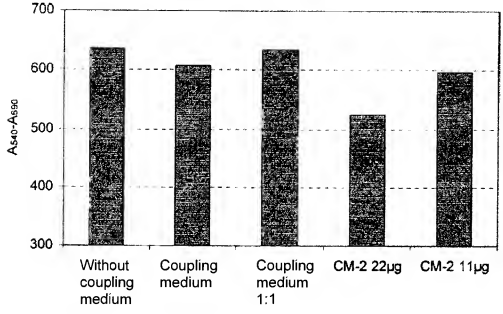
MTT test

– Trypsinize cells and resuspend in 10 mL RPMI complete medium (RPMI 1640, 10% FCS, 1% glutamine, 1% penicillin/streptomycin)
– Count cells and dilute to 1 x 10^6 cells per mL
– Pipette 50 µL cell suspension per well into a 96-well plate (leave first row empty); i.e., a cell count of 5 x 10^4 cells is present in each well
– Add 50 µL antibody (various dilutions in complete medium) per well
– Incubate the 96-well plate for 24 h or 48 h in a drying oven
– Pipette 50 µL MTT solution into each well
– Incubate plate for 20 min in the drying oven
– Centrifuge plate for 10 min at 2800 rpm and aspirate supernatant
– Add 150 µL DMSO per well and resuspend the cell pellet
– Determine absorption at a wavelength of 540 nm and 690 nm, using the ELISA reader.

[0042] MTT: Dissolve 5 mg/mL 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium (SIGMA) in PBS.
Antibody: CM-2
Cell line: COLO-206F (carcinoma of the colon)

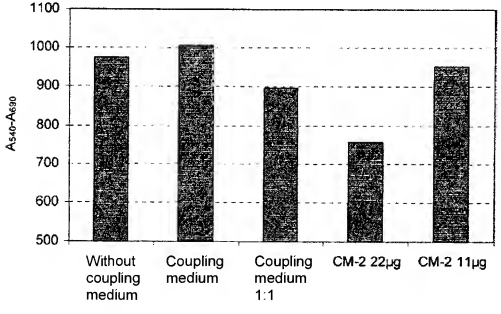
Incubation time: 24 h Cell line: COLD-[sic; COLO] 206F (carcinoma of the colon)

MTT test



Incubation time: 48 h

Antibody: CM-2



<110> Prof. Dr. Müller-Hermelink, Hans Konrad
Prof. Dr. Vollmers, H. Peter

<120> Human monoclonal antibody

<141> May 13, 2002

<211> 327

<212> DNA

<213> Homo sapiens

<220> Sequence of the variable region of the heavy chain (V_H) of CM-2 antibody (clone 98/81-33-154)

<221> V region

<222> (1)...(327)

<400>

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aaa aag ccc ggg gag tct ctg agg atc tcc tgt aag ggc tct gga tac agt ttt acc acc       60
Lys Lys Pro Gly Glu Ser Leu Arg Ile Ser Cys Lys Gly Ser Gly Tyr Ser Phe Thr Thr
 1               5                  10                  15                  20

tac tgg atc ggc tgg gtg cgc cag atg ccc ggg aaa ggc ctg gag tgg atg ggg atc atc      120
Tyr Trp Ile Gly Trp Val Arg Gln Met Pro Gly Lys Gly Leu Glu Trp Met Gly Ile Ile
            25                  30                  35                  40

tat cct ggt gac tct gat acc aga tac agc ccg tcc ttc caa ggc cag gtc acc atc tca      180
Tyr Pro Gly Asp Ser Asp Thr Arg Tyr Ser Pro Ser Phe Gln Gly Gln Val Thr Ile Ser
            45                  50                  55                  60

gcc gac aag tcc atc agt acc gcc tac ctg cag tgg agc agc ctg aag gcc tcg gac acc      240
Ala Asp Thr Ser Ile Ser Thr Ala Tyr Leu Gln Trp Ser Ser Leu Lys Ala Ser Asp Thr
            65                  70                  75                  80

gcc ata tat tac tgt gcg agg gag gtc tat act ggc cga aac tac tac tac tac ggt ctg      300
Ala Ile Tyr Tyr Cys Ala Arg Glu Val Tyr Thr Gly Arg Asn Tyr Tyr Tyr Tyr Gly Leu
            85                  90                  95                  100

gac gtc tgg ggc caa gga acc ctg gtc                                                 327
Asp Val Trp Gly Gln Gly Thr Leu Val
                105
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<110> Prof. Dr. Müller-Hermelink, Hans Konrad
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<120> Human monoclonal antibody

<141> May 13, 2002

<211> 330

<212> DNA

<213> Homo sapiens

<220> Sequence of the variable region of the light chain (V_L) of CM-2 antibody (clone 98/81-33-154)

<221> V region

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cag tct gcc ctg act cag cct gcc tcc gtg tct ggg tct cct gga cag tcg atc acc atc      60
Gln Ser Ala Leu Thr Gln Pro Ala Ser Val Ser Gly Ser Pro Gly Gln Ser Ile Thr Ile
 1               5                  10                  15                  20

tcc tgc act gga acc agc agt gac gtt ggt ggt tat aac tat gtc tcc tgg tac caa cag     120
Ser Cys Thr Gly Thr Ser Ser Asp Val Gly Gly Tyr Asn Tyr Val Ser Trp Tyr Gln Gln
                25                  30                  35                  40

cac cca ggc aaa gcc ccc aaa ctc atg att tat gat gtc agt aat cgg ccc tca ggg gtt     180
His Pro Gly Lys Ala Pro Lys Leu Met Ile Tyr Asp Val Ser Asn Arg Pro Ser Gly Val
                45                  50                  55                  60

tct aat cgc ttc tct ggc tcc aag tct ggc aac acg gcc tcc ctg acc atc tct gga ctc     240
Ser Asn Arg Phe Ser Gly Ser Lys Ser Gly Asn Thr Ala Ser Leu Thr Ile Ser Gly Leu
                65                  70                  75                  80

cag gct gag gac gag gct gat tac tac tgc agc tca aaa aga agc agc aac act cta gta     300
Gln Ala Glu Asp Glu Ala Asp Tyr Tyr Cys Ser Ser Lys Arg Ser Ser Asn Thr Leu Val
                85                  90                  95                  100

ttc ggc gga ggg acc aag ctg acc gtc cta                                          330
Phe Gly Gly Gly Thr Lys Leu Thr Val Leu
                105                 110
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Claims

1. Human monoclonal antibody containing heavy and light chain molecules, each having a region of constant composition from antibody to antibody and a region of variable composition from antibody to antibody, or a functional fragment thereof, **characterized in that**
– at least one variable region of the light chains has substantially the amino acid sequence stated in Appendix 2, and/or at least one variable region of the heavy chains has substantially the amino acid sequence stated in Appendix 1.

2. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 1 using the hybridoma technique, characterized in that

– the hybridoma cells are obtained by fusion
– of HAB-1 heteromyeloma cells and their subclones
– with B-lymphocytes removed from
– a lymphatic organ, preferably the spleen or the lymph nodes, of a cancer patient.

3. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 2, characterized in that
– the B-lymphocytes are removed from a patient with
– carcinoma of the stomach, colon, lung, pancreas, breast, prostate, or esophagus.

4. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 1, characterized in that
– the human monoclonal antibody or fragment thereof is produced using the recombinant method.

5. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 1, characterized in that
– the human monoclonal antibody or fragment thereof is produced by gene technology,
– using phage banks (phage display method).

6. Human monoclonal antibody or a functional fragment thereof according to one of Claims 1–5, characterized in that
– the structure of the constant region of the heavy chains corresponds to immunoglobulin M or G (IgM or IgG).

7. Human monoclonal antibody or functional fragment thereof according to one of Claims 1– [omission in source text; 6], characterized in that
the referenced functional fragment belongs to one of the groups
– V_L
– V_H
– Fv
– Fc
– Fab
– Fab'
– $F(ab')_2$.

8. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–7, characterized in that
– individual amino acid groups are
– substituted,
– and/or inserted,
– and/or removed.

9. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–8, characterized in that
– a first substance is coupled, in particular
– a radioactive substance,
– and/or a dye,
– and/or an enzyme,
– and/or an immunotoxin,
– and/or a growth inhibitor.

10. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–9, characterized in that
– a second substance is coupled, in particular for
– qualitative or quantitative detection,
– decreasing proliferation,
– producing apoptosis,
– avoiding metastasis formation of tumor cells.

11. Use of the monoclonal antibody or a functional fragment thereof according to one of the preceding claims for combating tumors, characterized in that the monoclonal antibody or functional fragment thereof is used for the
– diagnosis
– and/or prevention
– and/or treatment of the following tumors in particular:
– adenocarcinoma of the colon or endometrium.

12. Pharmaceutical agent, characterized in that
– the active substance thereof contains the referenced monoclonal antibody or functional fragments thereof.

13. Diagnostic agent, characterized in that
– the active substance thereof contains the referenced monoclonal antibody or functional fragments thereof.

No drawing sheet is appended

Morningside | Translations

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TRANSLATOR CERTIFICATION

I, Michael Magee, a translator fluent in the German language, on behalf of Morningside Translations, do solemnly and sincerely declare that the following is, to the best of my knowledge and belief, a true and correct translation of the document(s) listed below in a form that best reflects the intention and meaning of the original text.

MORNINGSIDE TRANSLATIONS

Michael Magee
Signature of Translator

Date: August 27, 2007

Description of Documents Translated:

DE 102 30516A1

(19) Federal Republic of Germany
German Patent and Trademark Office

(10) **DE 102 30 516 A1** 01/15/2004

(12) # Unexamined Patent Application

(21) Application No.: **102 30 516.1**
(22) Filing Date: **July 6, 2002**
(43) Date laid open for public inspection: **January 15, 2004**

(51) Int. Cl.[7]: **C07K 16/00**
A61K 39/395

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(72) Inventor:
Same as applicant

(56) Documents cited in the evaluation of patentability:
DE 41 07 154 A1

The following information has been taken from documents filed by the applicant

Request for search in accordance with §43, Paragraph 1, Clause 1, German Patent Act has been filed.

(54) Title: **Human monoclonal antibody**

(57) Abstract: Described is a human monoclonal antibody containing heavy and light chain molecules, each having a region of constant composition from antibody to antibody and a region of variable composition from antibody to antibody. According to the invention, at least one variable region of the heavy chains has substantially the amino acid sequence stated in Appendix 1. The invention further relates to a method for producing the antibody, use of the antibody for combating tumors, and a pharmaceutical agent and diagnostic agent containing the antibody.

Description

[0001] The invention relates to a human monoclonal antibody containing heavy and light chain molecules, each having a region of constant composition from antibody to antibody and a region of variable composition from antibody to antibody, or a functional fragment thereof. The invention further relates to a method for producing the antibody, and use of the antibody for combating tumors, and a pharmaceutical agent and a diagnostic agent which contain the antibody.

Background Information

[0002] Current methods for treating cancer include surgical removal of the tumor, radiation, and chemotherapy. A significant drawback of these methods is that they are not specifically targeted to the tumor cells. For surgical removal, for example, all of the tumor may not be removed, with the result that a new tumor develops and metastases possibly form which become established in other areas of the body. In the treatment of tumors by radiation or chemotherapeutic agents, the lack of selectivity often results in damage to healthy cells as well due to the agents used. The adverse result is that the doses of radiation or chemically active substances cannot be selected to be high enough that all the cancer cells are destroyed. A considerable portion of current cancer research is therefore devoted to finding more effective and in particular selectively active methods and agents for treating tumors.

[0003] Immunological studies have shown that cellular and humoral activity is measurable when the immune system cannot effectively combat malignant cells. However, this activity is not sufficient to destroy the tumor cells. A promising approach to combating tumors, therefore, is to isolate antibodies originating from the patient's immune response, suitably propagate the antibodies, and use them therapeutically.

[0004] One method from the prior art which uses this approach is the hybridoma technique, which is based on in vitro harvesting of cellular hybrids obtained by cellular fusion of normal lymphocytes with myeloma cells that are capable of unlimited viability and cell division. The hybridoma cells thus produced have the characteristics of both parent cells, and therefore have the ability of lymphocytes to produce antibodies, and also have the capability of myeloma cells for unlimited cell division and thus for producing antibodies in large quantities.

[0005] Each hybrid cell resulting from the fusion produces monoclonal antibodies whose specificity is determined by the original lymphocyte cell. The hybridoma cells are propagated, and the ones which produce antibodies of the desired specificity are then selected. Cultivation of this selection and isolation thereof results in highly specifically reactive antibodies which react only with a given antigenic determinant. Monoclonal antibodies which bind specifically to antigens of tumors thus offer promising opportunities for the diagnosis and treatment of tumor cells.

Object of the Invention

[0006] Thus, there is a need for such human monoclonal antibodies for improving the methods and agents for combating cancer. The object of the present invention is to provide a human monoclonal antibody, a method for production thereof, and diagnostic and pharmaceutical agents derived from the antibody which have a high specificity for antigens of various tumors, and which are therefore well suited for tumor-specific treatment and diagnosis.

[0007] This object is achieved according to the invention with regard to a human monoclonal antibody by the fact that at least one variable region of the light chains has substantially the amino acid sequence stated in Appendix 2, and/or at least one variable region of the heavy chains has substantially the amino acid sequence stated in Appendix 1.

[0008] From a chemical point of view, antibodies are immunoglobulin molecules. These molecules have two identical light chains and two identical heavy chains which are joined by disulfide bridges. Each of the chains has a region containing approximately 110 amino acids with a variable sequence, whereas the remainder of each chain has a region with a constant sequence. The variable regions of light and heavy chains each include multiple hypervariable regions which are responsible for binding of antigens. The specialized structure of the hypervariable regions thus determines the specific characteristics of the antibody.

[0009] Clinical tests have demonstrated that the structure of the referenced variable regions of the antibody according to the invention, depending on the given amino acid sequence, results in a high specific activity against the antigens of tumor cells under study. Since the antigens occurring on tumor cells are not present on normal cells, it is expected that the antibodies present exhibit little or no binding to normal cells.

[0010] It is essential to the invention that one of the variable regions of the light or heavy chains is substantially identical to the sequence according to the invention. By virtue of being substantially identical, the referenced regions predominantly match one another. The present invention encompasses minor modifications or substitutions of the chains, provided that the monoclonal antibody or the functional portion thereof maintains tumor-specific characteristics.

[0011] The majority of tumor-specific monoclonal antibodies of the prior art involve antibodies derived from mice. However, it is disadvantageous that such antibodies have very limited use, since mouse antibodies used in humans are recognized by the immune system as foreign proteins and may be neutralized before their therapeutic effect can be realized.

[0012] In contrast, the invention proceeds from human monoclonal antibodies, which do not have these limitations of use in human medicine. These antibodies contain sequences in the hypervariable chain regions which substantially correspond to those of human immunoglobulin. The antibodies, after recognition of the determinants or epitopes of the corresponding antigens, are thus able to bind in an unhindered manner to the affected cells without a defense reaction of the immune system. When the antibodies according to the invention are coupled to diagnostic and therapeutic agents, such antibodies are thus advantageously suitable for early recognition and effective treatment of various types of tumors.

[0013] The object is achieved with regard to the production method by the fact that the human monoclonal antibody is preferably produced by the hybridoma technique. According to one feature of the invention, for this purpose B-lymphocytes are removed from a lymphatic organ, preferably the spleen or the lymph nodes, of a cancer patient. As a result of the cancer, these lymphocytes are stimulated to produce antibodies which react specifically to the antigens of the tumor cells that are present.

[0014] Each lymphocyte is fused with a myeloma cell in vitro. According to the present invention, HAB-1 heteromyeloma cells and their subclones are used. The HAB-1 heteromyeloma cell is described in the literature by Falter, G et al., HAB-1, BrJCancer 62, 595-8 (1990). Similarly, subclones of the HAB-1 cell, referred to as HAB-1.X, may be used. The resulting cell clones have the same characteristics as the original B-lymphocytes for producing antibodies. The specificity of these antibodies is determined by the original lymphocyte cell. In the present case, this means that the antibodies produced by the cell clones also correspond to the antigens of the specific tumor that is present. After the cells are selected which synthesize antibodies of the desired specificity, these cells are cultivated, and each of the hybrid cells produces human monoclonal antibody in unlimited quantities.

[0015] According to one feature of the invention, in the provided method lymphocytes are removed from patients in particular with carcinoma of the

- stomach
- colon
- lung
- pancreas
- esophagus
- prostate
- breast.

[0016] In addition to production of the present human monoclonal antibody by the hybridoma technique, the invention also encompasses other production methods. Direct synthesis by the recombinant method, known to one skilled in the art, or production using the known phage bank method (phage display) are provided, in particular for the production of small functional fragments.

[0017] Propagation is performed by use of the known polymerase chain reaction (PCR).

[0018] The PCR method is known to one skilled in the art, for example from US 4,683,195. This method is used for the targeted propagation of a specific DNA fragment, and is advantageously employed when DNA segments are present only in trace quantities. By use of the method, one known DNA sequence from a number of similar sequences may be identified, and may be propagated in vitro in large quantities in a short time. A specialized DNA sequence may be propagated by a factor of approximately 100,000 over a time period of approximately 3 h.

[0019] When the present method is used for producing the monoclonal antibodies according to the invention or a functional fragment thereof, RNA of the hybridoma cells, which produce tumor-specific monoclonal

antibodies, is copied in vitro into complementary double-stranded cDNA, using reverse transcriptase. The cDNA, which codes functional fragments of the variable regions of the light and heavy chains, is then propagated using PCR. The PCR products are purified, extracted, and then cloned.

[0020] The composition of the constant region of the heavy chain of an antibody determines its isotype, and establishes the effector function of the antibody. For immunoglobulin, the constant region of the heavy chains is composed of one of the sequences, referred to in the literature as μ, γ, δ, α, or ε, and the constant region of the light chains is composed of one of the sequences κ or λ. The various compositions of the heavy chains result in the five immunoglobulin classes IgA, IgD, IgE, IgG, and IgM. The antibodies according to the present invention generally belong to class IgM, and light chains of class λ and κ may be present. The antibody may also have a composition of the IgG class.

[0021] The invention encompasses human monoclonal antibodies and functional fragments thereof. The functionality of the referenced fragments is characterized in that said fragments have the characteristics of the antibody. Such characteristics, for example, may be that the fragments have the ability to bind with antigens, or have specificity for tumor cells, or that the fragments have an effector function due to the composition of their constant region. According to one feature of the invention, fragments in particular are included which according to known nomenclature (e.g., Cell Biophysics, 22 (1993), pp. 189–224) belong to one of the groups V_L, V_H, Fv, Fc, Fab, Fab', $F(ab')_2$.

The V_L group comprises fragments which include the variable region, or the variable and constant region, of the light chains;

the V_H group comprises fragments which include the variable region, or the variable and constant region, of the heavy chains;

the Fv group comprises fragments which include the variable regions of the heavy and light chains or portions thereof;

the Fc group comprises fragments which include the constant regions of the heavy chains or portions thereof;

the Fab group comprises fragments which are larger than the fragments of the Fv group;

the Fab' group comprises fragments which are larger than the fragments of the Fab group;

the $F(ab')_2$ group comprises fragments which contain the variable regions of both heavy and both light chains or portions thereof, and which optionally contain the first constant regions of both heavy chains or portions thereof.

[0022] Specialized requirements for given applications may be met by use of the referenced fragments. According to one feature of the invention, the characteristics of the antibody or the functional fragments thereof may be modified by substituting and/or inserting and/or removing individual amino acid groups. These types of approaches may be used, for example, to modify the stability or the selectivity of the antibody or the functional fragments thereof while maintaining the global characteristics of the antibody, for example the ability to bind to tumor antigens.

[0023] According to the present invention, the antibodies or the functional fragments thereof may be joined to additional active substances. The fields of application for the present antibody may be significantly expanded by coupling of such substances. In particular, the human monoclonal antibody according to the present invention may be used for diagnostic methods for detecting tumor cells, and for therapeutic methods for combating tumor cells.

[0024] According to one feature of the invention, the following substances in particular are provided:

– a radioactive substance,
– and/or a dye,
– and/or an enzyme,
– and/or an immunotoxin,
– and/or a growth inhibitor,

whereby these active substances may be used for
– qualitative or quantitative detection,
– decreasing proliferation,
– producing apoptosis, or
– avoiding metastasis formation
of tumor cells.

[0025] The detection of tumor cells is frequently carried out using methods known by those skilled in the art as immunoassays, which are based on the antigen-antibody reaction. To obtain quantitative information from this reaction, the antibody according to the present invention is coupled to an easily detectable labeling substance.

The substance and the coupling are selected such that the immunological characteristics of the components are substantially maintained. The most well-known immunoassays are the radioimmunoassay, enzyme immunoassay, and fluorescence immunoassay. The diagnostic substances coupled to the antibodies allow sensitive and reliable methods for early detection of cancer.

[0026] The purpose of the referenced cytotoxic substances, for example, is to reduce the viability or the division capability of the tumor cells. Alternatively, the substances suppress DNA synthesis or cell division, or cause apoptosis of the cells or nonapoptotic cell death, and thus terminate growth of tumor cells or kill such cells.

[0027] By coupling the referenced substances with antibodies according to the present invention that are selectively active against cancer cells, it is possible to combat various types of tumors in a targeted and particularly effective manner. The invention provides in particular for the

– diagnosis
– and/or prevention
– and/or treatment

of the following tumors:

– carcinoma of the colon, pancreas, prostate, uterus, Fallopian tube, adrenal gland, and/or lung,
– squamous cell carcinoma of the esophagus or lung,
– carcinoma of the stomach,
– ductal carcinoma of the breast.

[0028] Lastly, the present invention also encompasses a pharmaceutical agent and a diagnostic agent, characterized in that the active substances thereof contain the referenced monoclonal antibody or functional fragments thereof. The referenced agents generally contain further additives, such as physiological solutions, solvents, glycols, oils, or similar substances known from the prior art.

METHODS, EXAMPLES, AND PARTICULARS

PM-2 antibody

Heavy chain (VH)

Amino acid sequence

see Appendix 1

DNA sequence

see Appendix 1

Light chain (VL)

Amino acid sequence

see Appendix 2

DNA sequence

see Appendix 2

Method 1

Immortalization of lymphocytes and primary testing of antibodies

[0029] For immortalization, the lymphocytes were fused with a variant of HAB-1 heteromyeloma according to standard protocol and cultivated. In summary, lymphocytes were fused with HAB-1 cells by means of PEG. The trioma were seeded on four 24-hole plates. The average growth rate was 80-90%, with 50% of the grown clones secreting immunoglobulins.

[0030] The first testing of the secreted human monoclonal antibodies was carried out using ELISA to determine the isotype. The next test was an immunohistochemical staining on cryosections of the autologous tumor.

Media required

- RPMI 1640 (PAA) without additives
- RPMI 1640 containing HAT supplement (HAT supplement, PAA) and 10% FCS, 1% glutamine, and 1% penicillin/streptomycin

Immortalization

- Wash HAB-1 (fusion partner) twice with RPMI without additives
- Centrifuge for 5 min at 1500 rpm
- Thaw frozen lymphocytes (from spleen or lymph nodes) and wash twice with RPMI without additives, and likewise centrifuge
- Take up each of the two pellets in 10 mL RPMI without additives, and count in the Neubauer counting chamber
- Fuse in an HAB-1 to lymphocyte ratio of 1:2 to 1:3
- Combine the cell pellets after the second washing, mix, and centrifuge for 8 min at 1500 rpm
- Carefully add the PEG (polyethylene glycol 1500, Roche), previously warmed to 37°C, dropwise to the pellet with slight rotational motions of the 50-mL test tube
- Gently resuspend, and then allow to rotate for exactly 90 sec in a water bath at 37°C
- Wash out the PEG with RPMI without additives (two full No. 10 pipettes)
- Centrifuge for 5 min at 1500 rpm
- Plate 24-well plates with 1 mL RPMI containing HAT supplement
- Dissolve the pellet in RPMI containing HAT supplement
- Pipette 0.5 mL of the cells into each of the 24 wells
- Place fusion plates in drying oven
- Replace medium weekly with RPMI containing HAT supplement

Method 2
Molecular characterization of the antibodies by sequencing

[0031] For sequencing of the monoclonal antibodies, cDNA was produced from total RNA (RNAse kit, Quiagen) from trioma (M-MLV reverse transcriptase, Gibco). The corresponding VH genes were then propagated by PCR amplification (Taq polymerase, MBI Fermentas). The PCR products were purified by gel electrophoresis and extracted. After cloning of the PCR products (pCR-Script Amp SK + cloning kit, Stratagene), the positive closes were sequenced (DyeDeoxy termination cycle sequencing kit, Applied BioSystems). The sequences were analyzed using DNASIS for Windows, Genebank, and V-Base databases (Vollmers et al., 1998).

Immunohistochemical characterization

[0032] Antibodies which react with the autologous tumor were tested on a panel of normal tissues and tumorous tissues in the immunoperoxidase test (see below for protocol) in order to obtain an overview of the antibody reaction and the antigen distribution.
[0033] Antibodies which react specifically with the tumor cells but not with healthy tissue were tested further, first against the same types of tumors from different patients, then against tumors from other organs, and finally against normal tissue. More detailed characterization of the antibody and antigen is not possible unless the reaction pattern of the antibody supports a conclusion of at least limited specificity for malignant tissue.

Immunoperoxidase staining on cryosections and cytospins

- Slides
- Allow slides to dry for at least 2 h after cutting
- Place slides in acetone for 10 min
- Allow to dry for 30 min
- Wash 3x with Tris-NaCl and then allow to stand in Tris-NaCl for 5 min
- Saturate with 100 µL milk powder (3% in PBS) for 15–30 min
- Wash 3x with Tris-NaCl
- 100 µL of the respective 1st antibody:
- for negative control, with RPMI

– for positive control, 1:50 CK8 with BSA/PBS or 1:10 CAM 5.2 with BSA/PBS (0.5% BSA in PBS)
– Incubate for 30 min
– Wash 3x with Tris-NaCl
– 100 µL of the respective 2nd antibody:
– 70% conjugated rabbit anti-mouse peroxidase with PBS + 30% human serum + 1:50 antibody
– 70% conjugated rabbit anti-human IgM peroxidase with PBS + 30% rabbit serum + 1:50 antibody
– Incubate for 30 min
– Wash 3x with Tris-NaCl
– Place slides in PBS for 10 min
– Dissolve 1 DAB tablet and 1 H_2O_2 tablet in 1 mL tap water
– Pipette 100 µL substrate onto the slides and incubate for 10 min
– Rinse with distilled water
– Place slides in hemalum for 5 min
– Rinse with water for 15 min
– Place slides in distilled water and mount with glycerin gelatin

IMMUNOPEROXIDASE STAINING ON PARAFFIN SECTIONS
Deparaffination:

– Xylene 1x, 5 min
– Xylene 2x, 5 min
– 100% ethanol 1x, 5 min
– 100% ethanol 2x, 5 min
– Methanol (70 mL) + H_2O_2 (500 µL), 5 min
– 90% ethanol 1x, 3 min
– 90% ethanol 2x, 3 min
– 80% ethanol 1x, 3 min
– 80% ethanol 2x, 3 min
– 70% ethanol 1x, 3 min
– 70% ethanol 2x, 3 min

Wash 1x with Tris-NaCl
Boil 300 mL distilled H_2O in pressure cooker and fill citric acid (pH 5.5) into insert, boil slides for 5 min
Block for 15 min with BSA/PBS, 150 µL per slide
Wash 1x with Tris-NaCl
Incubate 1st antibody, 150 µL per slide, for 2.5 h in a humidified chamber in drying oven
Wash 3x with Tris-NaCl
Incubate 2nd antibody, 150 µL per slide, for 45 min in a humidified chamber at room temperature
Wash 3x with Tris-NaCl
Allow to stand for 10 min in PBS
10 min DAB, 150 µL per slide
Wash 3x with H_2O, then wash 1x with distilled H_2O
Stain for 5 min with hemalum
Rinse with water for 10–15 min
Wash with distilled H_2O
Mount with glycerin gelatin

Staining of tumorous tissue

[0034] The tumorous tissues were stained to evaluate the number of carcinomas to which the antibody being studied showed a reaction.

PM-2
Staining of normal tissue

Tissue	CAM5.2	PM-2	IgM control
Breast	–	–	–
Prostate	–	–	–
Colon	–	–	–
Small intestine	–	–	–
Bladder	–	–	–

Staining of tumorous tissue

Tumor	Positive control	PM-2	IgM control
Pancreas	+	+	–
Lung (squamous cell)	+	+	–
Lung (adeno)	+	+	–
Breast	+	+	–
Stomach	+	+	–
Adrenal gland	+	+	–
Small intestine	+	+	–
Colon	+	+	–
Esophagus	+	+	–
Uterus	+	+	–
Fallopian tube	+	+	–
Prostate	+	+	–

[0035] Cell Death ELISA^{PLUS} (Roche, Mannheim The extent of apoptosis induction by CM-1 antibody was analyzed using Cell Death Detection ELISA^{PLUS}. This test is based on the principle of a quantitative sandwich enzyme immunoassay, using the peroxidase-conjugated mouse monoclonal antibodies directed against histone or DNA components. Following enzymatic conversion of a colorless substrate, the quantity of nucleosomes, and thus the relative number of apoptotic cells, present may be photometrically determined on the basis of the color intensity of the reaction product.

[0036] For this purpose, 100 µL of a cell suspension (1.0×10^5 /mL) of the various cell lines together with 100 µL of the undiluted or 1:1 diluted antibody supernatants in a 96-well plate were incubated for 24 h in a drying oven at 37°C and 7% CO_2. After completion of incubation the cells were centrifuged for 10 min at 200 g, the supernatant was aspirated, and 200 µL lysis buffer was added, resulting in cell lysis over the following 30 min at room temperature. After recentrifugation, 20 µL of the supernatant was transferred to each streptavidin-coated microtiter plate, and 80 µL of the immunoreagent (1/20 anti-DNA-POD, 1/20 anti-histone biotin, 18/20 incubation buffer) was then added by pipette. In addition, a positive control contained in the test kit and a blank assay were performed. After thorough mixing of the plates for 2 hours at approximately 250 rpm, followed by washing three times with incubation buffer (250 µL), 100 µL of the ABTS solution (1 ABTS tablet in 5 mL substrate buffer) was pipetted into each well. After remixing, the intensity of the antibody-induced apoptosis was indicated by an intense green precipitate. The color intensity was measured using an ELISA reader at λ = 415 nm against a reference wavelength of 490 nm, and on this basis the intensity of the antibody-induced apoptosis was calculated.

Cell Death ELISA
Antibody

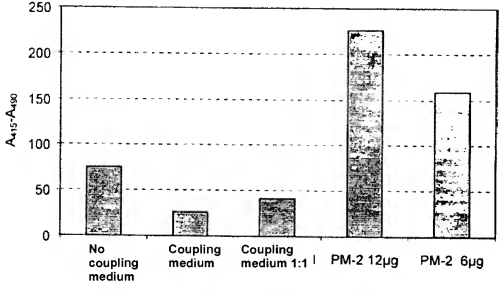
PM-2

Cell line

BXPC-3

Incubation time

24 h



Without: Negative control (RPMI 1460 medium)
PM-2: 6.12 µg/mL antibody supernatant

0037] After incubation for 24 hours, the PM-2 antibody being studied showed pronounced apoptosis indication [sic; induction] compared to the negative controls, the effect for PM-2 exceeding that of the negative control by a factor of 1.46.

MTT test

– Trypsinize cells and resuspend in 10 mL RPMI complete medium (RPMI 1640, 10% FCS, 1% glutamine, 1% penicillin/streptomycin)
– Count cells and dilute to 1 x 10^6 cells per mL
– Pipette 50 µL cell suspension per well into a 96-well plate (leave first row empty); i.e., a cell count of 5 x 10^4 cells is present in each well
– Add 50 µL antibody (various dilutions in complete medium) per well
– Incubate the 96-well plate for 24 h or 48 h in a drying oven
– Pipette 50 µL MTT solution into each well
– Incubate plate for 20 min in the drying oven
– Centrifuge plate for 10 min at 2800 rpm and aspirate supernatant
– Add 150 µL DMSO per well and resuspend the cell pellet
– Determine absorption at a wavelength of 540 nm and 690 nm, using the ELISA reader.

MTT

Dissolve 5 mg/mL 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium (SIGMA) in PBS.

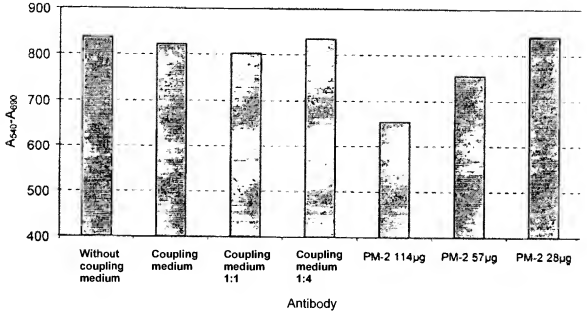
MTT test
Antibody

PM-2

BXPC-3 (carcinoma of the pancreas)

Incubation time

[0038] 24 h



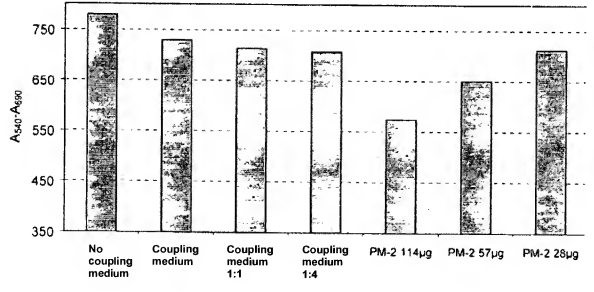
PM-2

Cell line

BXPC-3 (carcinoma of the pancreas)

Incubation time

48 h



<110> Prof. Dr. Müller-Hermelink, Hans Konrad
Prof. Dr. Vollmers, H. Peter

<120> Human monoclonal antibody

<141> May 13, 2002

<211> 321

<212> DNA

<213> Homo sapiens

<220> Sequence of the variable region of the heavy chain (V_H) of PM-2 antibody (clone 10/89-89)

<221> V region

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 1               5                  10                  15                  20

tgg gtc cgc cag gct cca ggg aag ggg ctg gag tgg gtc tca gct att agt ggt agt ggt       120
Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ser Ala Ile Ser Gly Ser Gly
                25                  30                  35                  40

ggt agt aca tac tac gca gac tcc gtg aag ggc cgg ttc acc atc tcc aga gac aat tcc       180
Gly Ser Thr Tyr Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                45                  50                  55                  60

aag aac acg ctg tat ctg caa atg aac agc ctg aga gcc gag gac acg gcc gta tat tac       240
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr
                65                  70                  75                  80

tgt gcg aaa ggt ggg gcc gaa ggc tgg tac gag tac tac tac tac tac ggt atg gac gtc       300
Cys Ala Lys Gly Gly Ala Glu Gly Trp Tyr Glu Tyr Tyr Tyr Tyr Tyr Gly Met Asp Val
                85                  90                  95                  100

tgg ggc caa ggg acc ctg gtc                                                          321
Trp Gly Gln Gly Thr Leu Val
                105
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<110> Prof. Dr. Müller-Hermelink, Hans Konrad
Prof. Dr. Vollmers, H. Peter

<120> Human monoclonal antibody

<141> May 13, 2002

<211> 348

<212> DNA

<213> Homo sapiens

<220> Sequence of the variable region of the light chain (V_L) of PM-2 antibody (clone 10/89-89)

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 1               5                  10                  15                  20

acc tgc acc ttg cgc agt ggc atc aat gtt ggt acc tac agg ata tac tgg tac cag cag      120
Thr Cys Thr Leu Arg Ser Gly Ile Asn Val Gly Thr Tyr Arg Ile Tyr Trp Tyr Gln Gln
            25                  30                  35                  40

aag cca ggg agt cct ccc cag tat ctc ctg agg tac aaa tca gac tca gat aag cag aag      180
Lys Pro Gly Ser Pro Pro Gln Tyr Leu Leu Arg Tyr Lys Ser Asp Ser Asp Lys Gln Lys
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ggc tct gga gtc ccc agc cgc ttc tct gga tcc aaa gat gct tcg gcc aat gca ggg att      240
Gly Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Lys Asp Ala Ser Ala Asn Ala Gly Ile
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Leu Leu Ile Ser Gly Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Met Ile Trp His
            85                  90                  95                  100

agc agc gct tgg gtg ttc ggc gga ggg acc aag ctg acc gtc cta ggt                      348
Ser Ser Ala Trp Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly
            105                 110                 115
```

Claims

1. Human monoclonal antibody containing heavy and light chain molecules, each having a region of constant composition from antibody to antibody and a region of variable composition from antibody to antibody, or a functional fragment thereof, **characterized in that**
at least one variable region of the light chains has substantially the amino acid sequence stated in Appendix 2, and/or at least one variable region of the heavy chains has substantially the amino acid sequence stated in Appendix 1.

2. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 1 using the hybridoma technique, characterized in that

the hybridoma cells are obtained by fusion
of HAB-1 heteromyeloma cells and their subclones
with B-lymphocytes removed from
a lymphatic organ, preferably the spleen or the lymph nodes, of a cancer patient.

3. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 2, characterized in that
the B-lymphocytes are removed from a patient with
carcinoma of the stomach, colon, lung, pancreas, esophagus, prostate, or breast.

4. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 1, characterized in that
– the human monoclonal antibody or fragment thereof is produced using the recombinant method.

5. Method for producing the human monoclonal antibody or a fragment thereof according to Claim 1, characterized in that
– the human monoclonal antibody or fragment thereof is produced by gene technology, using phage banks (phage display method).

6. Human monoclonal antibody or a functional fragment thereof according to one of Claims 1–5, characterized in that
– the structure of the constant region of the heavy chains corresponds to immunoglobulin M or G (IgM or IgG).

7. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–6, characterized in that
the referenced functional fragment belongs to one of the groups
V_L
V_H
Fv
Fc
Fab
Fab'
$F(ab')_2$.

8. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–7, characterized in that
individual amino acid groups are
substituted,
and/or inserted,
and/or removed.

9. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–8, characterized in that
one or more substances may be coupled, in particular
a radioactive substance,
and/or a dye,
and/or an enzyme,
and/or an immunotoxin,
and/or a growth inhibitor.

10. Human monoclonal antibody or functional fragment thereof according to one of Claims 1–9, characterized in that
a second substance is coupled, in particular for
qualitative or quantitative detection,
decreasing proliferation,
producing apoptosis, or
avoiding metastasis formation of tumor cells.

11. Use of the monoclonal antibody or a functional fragment thereof according to one of the preceding claims for combating tumors, characterized in that the monoclonal antibody or functional fragment thereof is used for the
– diagnosis
– and/or prevention
– and/or treatment of the following tumors in particular:
carcinoma of the colon, pancreas, prostate, uterus, Fallopian tube, adrenal gland, and/or lung,
squamous cell carcinoma of the esophagus or lung,
carcinoma of the stomach,
ductal carcinoma of the breast.

12. Pharmaceutical agent, characterized in that
the active substance thereof contains the referenced monoclonal antibody or functional fragments thereof.

13. Diagnostic agent, characterized in that
the active substance thereof contains the referenced monoclonal antibody or functional fragments thereof.

No drawing sheet is appended